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MONOGRAPHS ON MODERN CHEMISTRY

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THE CHEMICAL INVESTIGATION OF PLANTS

THE CHEMICAL INVESTIGATION OF PLANTS

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AUTHORISED TRANSLATION

Of the Third, improved and enlarged German Edition

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TRANSLATOR'S PREFACE

THE chemical investigation of Indian Medicinal Plants started by me in the Calcutta School of Tropical Medicine and the corresponding pharmacological and clinical studies instituted by the Professor of Pharmacology have led me to look for useful literature on the subject. For details, one is naturally obliged to refer to the vast mass of original papers published in various journals, German, French and English, but a beginner in this rather difficult subject is often very much embarrassed unless he finds some text which will guide him at the start. The present book provides such a beginner with the fundamental principles involved in the systematic chemical investigation of a plant and deals with the general methods followed for the isolation and purification of the various classes of chemical compounds met with in the vegetable kingdom. The book is thus a very useful guide not only for the pharmaceutical or the agricultural chemists but also for the various research workers engaged in the study of plant products from the medicinal, pharmacological or purely scientific points of view, and I shall feel amply compensated for my labours if this important book is well appreciated in the English-speaking countries.

I am much indebted to Dr. F. M. Haines of the University of London East London College for valuable help in the preparation of the translation.

S. GHOSH

August 1930

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INTRODUCTION

The chemical composition of a plant is completely ascertained only when the nature and quantity of all the chemical individuals of which it is composed are known. The execution of an investigation planned in this sense is a problem of which the complete solution, even in the present high standing of the natural sciences and of chemistry in particular, still offers great and in many respects insuperable difficulties at the start, since it has not as yet been possible to split up a considerable number of substances, such as enzymes and membrane matter, into chemical individuals. In most phytochemical investigations, however, such a thorough analysis is neither aimed at nor is it necessary, and, above all, investigations of this kind are undertaken with various motives. The pharmacist and the pharmacologist have often quite different aims in view from the plant physiologist and the agricultural chemist. Most phytochemical investigations, as they are undertaken in pharmaceutical, chemical and pharmacological laboratories, have for their object the preparation of pure plant-products which have medical, technical or scientific importance and interest, and the investigation of their composition in more or less detail.

It is far more difficult to ascertain the organic constituents of a plant than its inorganic components. While in the investigation of the latter only a relatively small number of known elements and compounds come into consideration, the number of organic substances occurring in plants, although they are composed of only a few elements, is enormously large. The properties of most of the substances, the preparation of which is the aim of the investigation, are unknown, and the decision whether the isolated final substances are chemical individuals or not is not always an easy matter.

Moreover, the plant chemist is often faced with the question whether the substances obtained in the course of his investigation were originally present in the plant or whether they are only decomposition products. For the action of heat, atmospheric oxygen, enzymes, and possibly also of the dissolved substances on one another which can seldom be entirely excluded in this work, and also the frequently acid or alkaline solvents may often produce changes in the plant products originally present [1] which even with great expense of trouble and sagacity are very difficult to detect.

To this is added the fact that the composition of a plant or even a portion of the plant is not the same in all circumstances. Seasonal changes, differences in habitat and chemical nature of the soil and artificial interference by man in cultivation can produce far-reaching changes in the qualitative as well as the quantitative composition of the plant. Thus the glucoside content of the leaves of digitalis collected during the same period of vegetation varies a good deal if they originate from different places. It is also a well-known fact that the alkaloid content of the cinchona barks of Java has been considerably increased by measures for cultivation—pre-eminently, of course, by breeding.

Finally, quantitative estimations frequently give only approximate results. For example, the methods used in pure science for the estimation of tannins, proteins and membrane-matter still leave much to be desired.

In view of the large variety of substances occurring in plants one can easily understand that a systematic process for their identification, such as that used in inorganic chemistry, does not and indeed cannot exist to the same degree of perfection as long as complete investigations of a considerable number of plants from all families of the vegetable kingdom continue

[1] It is frequently especially difficult to prove after the isolation of an optically inactive substance with an asymmetric molecule whether or not the method of preparation has racemised it.

to be unavailable. For it is not otherwise possible to draw up a method of investigation following which one would be sure to find out all the substances occurring in the vegetable kingdom in an unchanged condition—certainly a very narrow net to catch all the substances sought for. When, in spite of this, one such process (as aimed at in the outlines of Dragendorff's method) is given, it is merely given in the belief that the beginner can proceed better with the intricate relations of plant chemistry with such a method than without any such help, provided only that he realises it must not be followed slavishly in all circumstances. The plant chemist must always possess a large measure of the faculties of shrewdness, observation and common sense if nothing is to escape him and justice is to be done to the problem in every case.

On the other hand, there are circumstances which tend to simplify the task of the plant chemist. In the large medley of compounds which enter into the composition of plants, groups of substances can be distinguished the individuals of which possess common properties. For the unknown members of these groups, which occur, for example, in the alkaloids, saponins, tannins, sugars and proteins, he searches by using methods of investigation which have already served for the detection of known members of the same group, or he tries a new method founded on a careful consideration of the common properties of all the known members of the group. A large number of plant products, the properties of which are known, are widely distributed in the vegetable kingdom. It is therefore easy (though it may often be only a matter of secondary importance) to establish their presence among the objects of investigation. Such substances are chlorophyll, grape sugar, cellulose, starch and similar substances.

It is often easier to establish the absence of a member of a certain group than definitely to prove its presence. If, for example, the concentrated acid extract of a plant gives no

precipitate with the most usual alkaloid reagents, then the presence of an alkaloid is excluded.

The way in which a plant is used often affords a clue for the investigation. Foods contain digestible carbohydrates, fats or proteins; in plants used for condiments substances with basic properties are frequently found, and in vegetable washing materials the presence of saponin-like glucosides may be assumed.

In the investigation of plants with marked physiological action it is advantageous to carry out biological experiments. In this way it can be determined whether the extracts as well as the isolated substances possess the specific action.

The position of plants in the natural scheme of classification often gives information concerning the nature of the substances the presence or absence of which is to be expected in the plants. Thus, in the Papaveraceae one may expect to meet with protopin, their characteristic and principal alkaloid. The presence of alkaloids in the Solanaceae and of amygdalin in the seeds of the Prunaceae is expected as a matter of course. On the other hand, one does not expect to meet with a cyanogenetic plant in the family of the Umbelliferae.

If several substances of the same kind, perhaps alkaloids or glucosides, occur in a family or even in one and the same plant, then it may be assumed from accumulated experience that their chemical compositions will be very similar. Thus all the cyanogenetic glucosides of the Prunaceae are derived from benzaldehyde cyanhydrin, and the same nucleus is present in all the Columba alkaloids.

Rules

1. Examine with a microscope every solid substance isolated in order to determine whether or not it is outwardly homogeneous.

2. Before analysis purify every substance until there is not the slightest doubt about its being a single substance. The

analysis of an imperfectly purified substance is the most frequently committed mistake in plant chemical investigation.[1]

It may, however, be explicitly pointed out that the preparation of pure plant products, even in the case of substances which crystallise well, is often attended by great difficulties, since it frequently happens that a mixture of substances obstinately crystallises together. Special care is necessary with amorphous substances.

3. Never analyse any substance before it has been tested qualitatively. Never omit to test the substance for nitrogen, sulphur and phosphorus and to determine the constituents of the ash.

[1] The practice of giving well-sounding names to impure substances, of conducting careless investigations and building up hypotheses as audacious as they are false upon the doubtful results should be altogether abandoned.

SECTION I: GENERAL

A Short Outline of the History of Plant Chemistry

If plant chemistry is regarded as the art of resolving plants into their chemical constituents, or at least as that of isolating the most important components in the form of pure chemical compounds, then the apothecary, C. W. Scheele, may be regarded as the founder of modern plant chemistry (*1*). The number of plant products known before Scheele's time was very small, and perhaps no single substance had been prepared in perfectly chemically pure condition. The Romans and Greeks, however, knew cream of tartar of their own preparation, and in Eastern Asia, Borneo camphor and Lauracean camphor were known at an early date, the first needing only to be scraped out of the trees. To these were added a few substances which were obtained by the easy process of dry distillation. Thus George Agricola in 1546 observed among the products of the dry distillation of amber a substance which we now designate as succinic acid, and Alexander Pedemontanus in 1560 obtained crystals of benzoic acid from benzoin in the same manner. The art of steam distillation, already highly developed in the sixteenth century, and the preparation of essential oils specially advanced by Valerius Cordus, led to mixtures from which a crystalline substance separated out but rarely. The thymol of thyme oil seems, however, to have been observed as early as the seventeenth century, and Valerius Cordus described the solidification of anise oil.

In quite a different manner, Angelus Sala, who had already prepared cream of tartar and cane sugar in pure condition, succeeded in the discovery of salt of sorrel about the middle of the seventeenth century while he was concentrating sorrel sap which had been clarified by white of egg.

Of the two chief methods of investigation which were at the disposal of phytochemists at that time, viz. dry distillation and extraction with solvents, the former process came above all to the forefront when the Académie des Sciences in Paris, soon after its foundation (1666), took up the investigation of plants on the largest scale that had yet been undertaken. Its members, Duclos and Dodart, who worked on the theoretical side, were of opinion that in order to acquire fundamental knowledge of a plant one must use force and decompose it completely by fire. Thus, during the next twenty-five to thirty years, no less than 1400 plants and plant products were investigated by the method of dry distillation. The result was that approximately the same products were obtained from all plants, and thus the knowledge attained was that this process was useless for ascertaining the real components of plants—a conclusion which penetrated to plant chemists in general but very slowly. In the eighteenth century, the extraction method again came to the forefront with Boulduc, Boerhaave and Neumann, without, however, any noteworthy results. The best work of this epoch was that of the Berlin apothecary Marggraf, that of most consequence being carried out in 1747 when he demonstrated the preparation of cane sugar from indigenous plants.

It was Scheele who first raised phytochemistry out of this stagnation. Even in his first investigation, organised at the Stockholm Académie, he described (1769) the discovery of tartaric acid. He had obtained it by decomposing cream of tartar with chalk and then setting the tartaric acid free from the calcium tartrate by means of sulphuric acid. In a similar way he also prepared citric acid from lemon juice. Then followed the discovery of oxalic acid and of malic acid, the proof that the crystals found in Rhubarb and in many other drugs were identical with calcium oxalate, and also the preparation of benzoic acid from benzoin by means of the lime method. Even in the last year of his life (1786) he discovered

a new vegetable acid, gallic acid, which he observed during the spontaneous decomposition of an infusion of gall-nuts. These works of Scheele, though small in number and extent, greatly exceeded in value all the work carried out before his day in the field of plant chemistry. In the place of fruitless speculation, indulged in by so many of his predecessors, he conducted well-planned and carefully prosecuted experimental investigations. Instead of aqueous and alcoholic extracts which people before him and even long after him would represent as isolated individual compounds out of the plants, he prepared his acids as chemically pure substances with correctly defined properties. He showed conclusively for the first time that the components of plants ought to be prepared in a systematic manner in the form of chemical individuals and that this was possible (*1*). The influence of Scheele has never disappeared from plant chemistry and can be traced in the following epoch, the epoch of the discovery of vegetable bases (*2*).

In the beginning of the nineteenth century the French apothecary Derosne and his German colleague Sertürner were busy with the investigation of opium and obtained the same crystalline substance, Derosne in 1804 and Sertürner two years later. Derosne had also established that this substance was alkaline in reaction. But as he had used potash as a precipitant he supposed that the alkaline reaction was to be ascribed to contamination with this substance. It was Sertürner who first showed in 1806 that the alkaline reaction of the substance isolated from opium, to which Gay-Lussac gave the name morphine, was due to the substance itself, and that this substance was a base capable of forming a salt as expressed by Sertürner. The name alkaloid was applied to these substances by Meiszner in 1818, and other representatives of the class were found even during the same year. Narcotine, which was discovered by Derosne in 1803, was again prepared by Robiquet in 1817. The Portuguese Gomez isolated basic substances

from cinchona bark as early as 1811, in 1817 Pelletier and Magendie isolated emetine, in 1818 Pelletier and Caventou discovered strychnine, and the same year saw the discovery of veratrine by Meiszner. Naturally, the discovery of Sertürner gave the impetus for the investigation of all the important medicinal plants for alkaloids. The years 1817-1837 may be regarded as the most brilliant period in the discovery of alkaloids as the above review and the following summary (*3*) will show.

Year	Alkaloid	Discoverer
1819	Brucine	Pelletier and Caventou
	Piperine	Oersted
	Delphinine	Brandes and Lassaigne
1820	Quinine	Pelletier and Caventou
	Caffeine	Runge
	Solanine	Desfosses
1824	Surinamine	Hüttenschmid
	Chelidonine	Godefroy
1825	Sinapine	Henry and Garot
1826	Corydaline	Wackenroder
	Berberine	Chevalier and Pelletan
	Coniine	Giesecke
1828	Nicotine	Posselt and Reimann
1829	Curarine	Roulin and Boussaingault
1830	Buxine	Fauré
1831	Atropine	Mein
1832	Codeine	Robiquet
	Narceine	Pelletier
1833	Quinidine	Henry and Delondre
	Hyoscyamine	Geiger and Hesse
	Aconitine	Geiger and Hesse
	Colchicine	Geiger and Hesse
1834	Bebeerine	Rodie
1835	Thebaine and Pseudomorphine	Pelletier
1837	Jervine	Simon
	Harmaline	Goeben

The enormous advance of phytochemistry, which resulted from the discovery of Sertürner, led to the discovery of many non-alkaloidal vegetable principles during this period, notably that of amygdalin in 1830 by Roubiquet and Boutron-Charlard. The famous investigations of Liebig and Wöhler, published in 1837, became the starting-point for the creation of the group of glucosides [1] after they had established the fact that glucose was one of the products of their hydrolysis. This work is also significant in the history of the vegetable enzymes.

The following table (*4*) gives a survey of the discovery of the most important glucosides :

Year	Glucoside	Discoverer
1822	Daphnin	Gmelin and Baer
1824	Antiarin	Pelletier and Caventou
1827	Sinalbin	Henry and Carot
1828	Digitalin	Dulong
	Hesperidin	Lebreton, Brandes
1830	Salicin	Leroux
	Populin	Braconnot
	Aesculin	Minor and Brandes
	Amygdalin	Roubiquet and Boutron-Charlard
1832	Gypsophila-Saponin	Bussy
1835	Phloridzin	de Koninck
1840	Sinigrin	Roubiquet and Bussy
1841	Quercitrin	Bolley
	Syringin	Millet
1842	Rutin	Weisz
1843	Apiin	Braconnot
1852	Arbutin	Kawalier
1857	Fraxin	Salm-Horstmar
1861	Coniferin	Hartig
	Glycyrrhizin	Gorup and Besanez

[1] Laurent (1852) was the first to collect together all the substances which gave sugar on hydrolysis into a special group which he called glucosamides and Berthelot termed them later saccharides. I do not know who originated the term glucoside.

Year	Glucoside	Discoverer
1862	Gentiopicrin	Kromeyer
1866	Naringin	de Vrij
1868	Rhinanthin	Ludwig
1872	k-Strophanthin	Fraser
1874	Digitoxin	Schmiedeberg
1875	Digitonin	Schmiedeberg
1877	h-Strophanthin	Hardy and Gallois
1897	Linamarin	Jorissen and Hairs

The systematic work on essential oils, and with it the preparation of their individual constituents in a pure state, began first in the nineteenth century. This was made possible by the progress of the technique of distillation (fractional distillation, vacuum distillation), as also by the progress of organic chemistry, which enabled the separating out of single components by causing them to form chemical compounds, *e.g.* the phenols as phenolates, the aldehydes and ketones as bisulphite compounds and the terpenes by the method of preparing them from additive compounds with halogens, halogen acids, etc., especially developed by Wallach.

The first pure compounds prepared from this group were menthol, thymol and eugenol.

Further progress in phytochemistry may be expected through *microchemistry*, which has so far really helped our subject only in the analysis of vegetable products.[1] If the methods of micro-extraction, micro-distillation, micro-sublimation, etc., can be successfully developed, the present tedious investigation of plants will be very much simplified and phytochemical investigation will receive a fresh impetus.

[1] The masterly microchemical determinations of the constitution of alkaloids carried out by E. Späth and his students (Vienna) may be specially referred to here.

General Remarks on some of the Procedure necessary for the Preparation of Plant Constituents

The preparation begins with extraction or, in the case of volatile substances, with distillation. Sublimation can be used in special cases as in the preparation of benzoic acid from gum bezoin.

The use of a percolation apparatus for extraction in the cold and of extraction apparatus for hot extractions considerably simplifies the work. If the application of heat cannot be avoided during the extraction a water-bath should be used wherever possible.

The length of the extraction and the number of times it is performed depend upon the properties of the substance to be isolated and upon the method of investigation. As a general rule the extraction should be continued until the material is fully exhausted. In working with extraction- or percolation-apparatus this point can often be detected when the solvent, originally coloured, runs out colourless. If the extract is colourless from the start a small portion should be evaporated from time to time on a watch-glass. If the liquid leaves behind no solid residue the extraction is complete. In the extraction of fats the liquid is allowed to act upon a piece of paper, and a rough measure of the progress of the extraction can be obtained from the grease spot left behind. In the case of tannins the extraction is continued until the ferric chloride reaction (see p. 20) is no longer observed. With saponins the foam reaction can be used (see p. 21); with alkaloids the very sensitive alkaloid precipitants. The presence of non-alkaloidal substances with a bitter or other taste can be detected by tasting. The residue from the extraction should be pressed out since it always retains some of the solvent.

The subsequent filtration of the extract, when there is no centrifuge, is often a very tedious matter. The best thing to do is to allow the extract to settle down completely and decant

off the clear liquid before putting the turbid residue upon the filter. As *clarifying agent*, one can use talc or kieselgur, which carry down the fine suspensions to the bottom when the liquid is shaken with them. In the case of a slimy fluid the addition of alcohol is often helpful.

An excellent process of filtration is that of Pukall, in which the liquid is sucked through an earthenware cell (Pukall's cell) by means of a water-pump. It is often useful when all the other methods of filtration fail.

The filtration with Jena-glass filters and with plunging suction filters may also be referred to. For this, compare A. Heiduschka and F. Muths, *Pharmazeut. Ztg.*, 72, p. 1614 (1927).

The evaporation should, whenever possible, be done *in vacuo* in order to avoid oxidation and decomposition favoured by a higher temperature.

If an alcoholic liquid is to be evaporated in order to take up the residue with water, care should be taken to remove the alcohol completely, as otherwise chlorophyll and other alcohol-soluble but not water-soluble substances which are usually present go into the aqueous solution and create such a turbidity that its clarification by filtration may be no longer possible. The same holds good for the reverse case. If a turbidity is formed in spite of this it may be often removed by shaking with ether.[1]

If the decolourisation of the extract has been done by animal charcoal, the charcoal should be carefully boiled afterwards with different solvents since it may take up alkaloids and other substances besides adsorbing the colouring matter.

In favourable cases crystalline products may be obtained directly during the preparation, which have only to be purified by charcoal and recrystallised in order to obtain them in a pure condition. In the majority of cases, however, the

[1] Chlorophyll in colloidal solution can be best removed by shaking with ether after the addition of common salt.

crystallisation is not such an easy matter and the liquor may have to be kept as long as a month *in vacuo* over sulphuric acid before the crystals appear.[1] If the substance is known, or if only a few substances are concerned, the crystallisation can be started by sowing with a crystal (inoculation).

If crystallisation results on inoculation the substance is in most cases identified thereby, since crystallisation can only be effected by the same or an isomorphous substance, and in the province of plant chemistry the latter rarely come into consideration. The *inoculation* can be performed in various ways (*5*).

1. **Bourquelot's Process.** The inoculating crystal is rubbed on a slide and a drop of the syrup is placed upon the spot rubbed. A cover-glass is placed over it and the slide put under a glass bell-jar. If the result is positive crystals appear sooner or later, first on the part rubbed and then in other places. If sufficient crystals are formed thereby they are scraped off and used for sowing the main bulk of the syrup.

2. **Denigès' Micro Method.** The inoculating material is first prepared in the following way: About 1 mg. of the finely powdered crystalline substance is placed in a small heap on the middle of a slide. A small drop of water (about 1 cm. in diameter) [2] is put upon it, warmed very carefully over a small flame, and the slide is then quickly removed to allow the solvent to evaporate spontaneously. If it has been so far dried that the residue forms a varnish (not too dry), the drawn-out end of a glass rod may be dipped into a crystalline powder of the same substance and the residue rubbed with it. Micro-crystals are thus obtained along the lines rubbed.

At the most 0·1–0·2 mg. of the substance to be tested is then placed upon a slide and dissolved in a small drop of water

[1] The substances which crystallise with difficulty include among others malic acid, sugars and polyhydric alcohols.

[2] It is here assumed that when crystallisation takes place with difficulty the substances concerned are always water-soluble ones.

the diameter of which should not exceed 3–4 mm. It is carefully evaporated as described above to a soft varnish and rubbed lightly in different directions with the rough drawn-out end of the glass rod used in the first preparation. It is then moistened with a drop of a mixture of equal volumes of acetone and glacial acetic acid and the drop is allowed to evaporate. The moistening and evaporation treatments are then repeated, and after the evaporation of the solvents it is examined for crystals under the microscope (without using a cover-glass).[1]

Water-soluble substances which are insoluble in alcohol can be crystallised by dissolving them in water, adding alcohol until the solution is turbid, then removing the turbidity with water and keeping the solution in a desiccator over quicklime. The water is taken up by the lime and the liquid becomes proportionately richer in alcohol.

A crystalline substance is generally considered to be pure only when repeated recrystallisation no longer alters its melting-point (cf. p. 5).

It is more difficult to establish the purity of a substance which melts with decomposition or is amorphous or liquid. The method of testing and purifying liquids which boil without decomposition is to submit them to *fractional distillation* and to collect separately the fractions which distil over at one temperature or within a short range of temperatures. This operation, even if frequently repeated, does not always lead to the desired end, since mixtures of liquids may also have constant boiling-points. In such cases attempts may be made to prepare *derivatives* of the substances, since it may be far easier to separate these than their mother-substances. One such process has been proved to be especially useful in the case of terpene-like components of essential oils and is often used for the separation of non-volatile substances. Alkaloids

[1] See also G. Denigès' " Qualitative Analysis by Microcrystalloscopy " in *Fortschritte der Microchemie* (1928), p. 21.

can, for this purpose, be converted into salts, glucosides into acetyl-compounds, acids into esters, and so on.

A far more important process of separation depends upon the principles of fractional precipitation and *fractional solution*. To carry out the latter method, the amount of the solvent required to dissolve a small portion of the substance completely is first of all ascertained. The substance is then treated five times with one-fifth of the amount of liquid required to dissolve it completely (or ten times with one-tenth of this amount) and the properties of the substance obtained by the concentration of each solution are tested, especially the melting-point and elementary composition. *Fractional precipitation* is also done in a similar way. The amount of the precipitating agent necessary to precipitate all the substance present in the solution is ascertained and the latter then precipitated with a fifth of the amount of precipitating agent required for complete precipitation five times (or, with a tenth of the amount ten times) and the precipitate filtered off after each precipitation. If the substance so treated consists of two substances behaving differently with the solvent and the precipitating agent then the differences between the first and last fractions obtained by following the above operations can be ascertained and a separation accomplished by repeating the fractionation.

Sublimation in high vacuum can be used in many cases for the purification of substances. For an apparatus suited to this purpose (obtainable from Hauff & Buest, Berlin NW 6, Luisenstrasze 67) see H. Dieterle and B. Holländer, *Archiv d. Pharmazie* 265 (*Ber. deutsch. pharmazeut. Gesellsch.* 37), 118, 1927.

Among micro-apparatus may be mentioned: the *Micro-Extraction Apparatus* of G. Klein, the *Micro-Distillation Apparatus* of Gawalowski-Klein, and the *Micro-Sublimation Apparatus* of Klein-Werner. Illustrations of these apparatuses will be found in G. Klein: *Histochemie im Dienste der Waren-*

kunde, in Grafe's *Handbuch der organischen Warenkunde* ; and of the last-mentioned apparatus also in *Zeitschr. f. physiolog. Chem.* 143, 141, 1295.

If a solid substance is believed to have been prepared in a pure condition, it should be first tested for the presence of inorganic constituents. On heating on a platinum foil the organic portion will burn off while the inorganic matter will remain as ash.[1] If there is an ash, one should try to free the substance from the inorganic constituents in case these do not enter into its constitution. To obtain it free from ash, the substance can be fractionally dissolved or precipitated. For non-dialysing water-soluble substances, the process of dialysis can be employed (cf. p. 115), free acid being used to facilitate the process providing this can be done without injuring the substance.

One should also determine whether the substance contains nitrogen, phosphorus or sulphur. To test for *nitrogen*, 0·05 to 0·1 gm. of the substance is heated in a dry test-tube with sodium and the product of the reaction, in which the nitrogen originally present in the substance occurs as sodium-cyanide, is extracted with water. The cyanide in the solution is recognised, as it becomes Prussian-blue on boiling for a few minutes with ferrous sulphate and ferric chloride and then acidifying with HCl.[2]

The *sulphur* can also be detected by heating with Na. Sodium sulphide is formed thereby, which can be demonstrated by the purple-violet colour with sodium nitroprusside or by the black stain obtained on a bright silver coin. Sulphur can also

[1] On micro-incineration see A. Schoeller : *Ber. d. Dtsch. Chem. Ges.* 55, 2191, 1922.

[2] If a substance contains sulphur, it is better to test for nitrogen, *i.e.* for the cyanide formed by the action of sodium by the thiocyanate reaction. The filtered aqueous extract obtained after the sodium fusion is mixed with a little yellow ammonium sulphide and concentrated on the water-bath but not evaporated to dryness. It is cooled, strongly acidified with nitric acid, filtered and the filtrate treated with a solution of ferric sulphate or iron-alum. If nitrogen was originally present, a stable blood-red colour is obtained.

be identified by oxidising it to sulphuric acid by heating with fuming nitric acid or with caustic potash and potassium nitrate and testing with barium chloride.

By the same oxidation reaction the *phosphorus* is converted into phosphoric acid, and this is identified by the precipitate obtained with magnesia mixture or ammonium molybdate.

These preliminary tests are followed by the determination of the *elementary composition* of the substance by combustion and, where possible, by determination of its *molecular weight*. The *solubility* relations of the substance should also be determined and its *behaviour with reagents*.

Preliminary Tests

a. **Micro-sublimation.** A little of the powdered substance is heated in Tunmann's apparatus [1] and the slide on which the volatile substance sublimes is frequently changed.

In many cases it is better to sublime *in vacuo*. For this purpose the simple apparatus illustrated here (Fig. 1) can be used. A test-tube containing the material, powdered as finely as possible, and over it a little glass wool, is tightly connected by means of a piece of rubber tubing with a bent glass tube. The lower end of this tube carries a round cover-glass, fixed to it by means of a little glycerine and having a hole in the middle. The whole apparatus is placed in a sand or oil bath and heated while a vacuum is produced inside by means of a water-pump. The sublimate is deposited on the cover-glass and can be conveniently examined upon it directly under a microscope. Larger quantities of material can be worked in E. Tiedemann's Vacuum Sublimation apparatus (*6*). (It can

[1] Tunmann's apparatus (figure in O. Tunmann, : *Pflanzen Mikrochemie*, and L. Rosenthaler : *Qualitative Pharmazeutische Analyse*) consists of an iron ring fixed upon a stand on which is placed a sheet of asbestos. Upon the latter a little of the substance to be heated is placed by means of a piece of slide. To catch the sublimate a slide is used and is allowed to rest on the asbestos sheet over the substance to be heated, being supported on a small piece of glass or a small asbestos plate.

be procured from the research laboratory of Siemens and Halske, A.-G., and of Siemens-Schuckertwerke, G. m. b. H., Berlin-Siemens-stadt).

The apparatus (Fig. 2) consists of a strong-walled glass cylinder, bulged at the bottom and provided with a side tube for creating

Fig. 1.

Rosenthaler's Apparatus for Vacuum Sublimation.

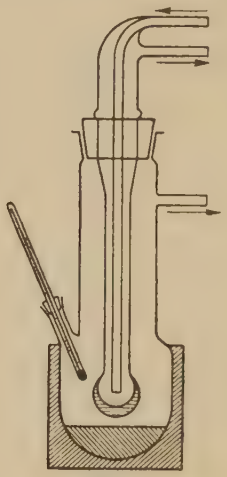


Fig. 2.

E. Tiedemann's Apparatus for Vacuum Sublimation.

a vacuum, and, finally, with a tube for a thermometer. Through a rubber stopper in the neck of the cylinder passes a water-cooling device which consists of a glass or metal tube blown out at the bottom in the shape of a pear.

To use the apparatus finely powdered and dried material for sublimation is placed in the lower part, the cooling arrangement and thermometer inserted, the apparatus evacuated with a high-vacuum pump (*e.g.* an oil pump) and heated slowly in an oil bath (or an electric bath) to a suitable temperature. The sublimate is thus obtained as a thick crystalline cake.

The sublimate formed is examined under the microscope and tested for reaction, solubility, presence of alkaloids (see p. 21) and *oxy-methyl-anthraquinones* (red colour with KOH solution).

The process does not, however, allow one to determine

whether or not the sublimed substance was originally present in the material investigated.

b. A small portion (5–10 gms.) of the substance to be investigated is warmed with water on the water-bath at 50–60° C. and the cooled, filtered aqueous extract is tested.

(1) For its reaction. An acid reaction, which is usually given, shows the presence of acids, acid salts, tannins or phenolic substances.

(2) With ferric chloride. Colourations are given with many substances ; blue or green colourations especially with tannins.

(3) With lead acetate. If there is a precipitate, the solution should be precipitated completely with lead acetate (the filtrate should not give any more precipitate with lead acetate) and tested with basic lead acetate to discover if this causes any precipitate with the filtrate.[1]

Many acids, tannins, plant-mucins and proteins are precipitated by lead acetate, while other substances like gum arabic and a few glucosides are precipitated first by basic lead acetate.

(4) By heating with a freshly prepared Fehling's solution. If there is a precipitate of cuprous oxide, reducing substances, like glucose, are present. If there is no reduction, the liquid is heated after the addition of HCl then made neutral [2] or alkaline with NaOH, and heated again with Fehling's solution. If it now reduces, the liquid contains one or more substances which produce by cleavage a reducing substance on boiling with acids, *e.g.* glucosides or disaccharides.

c. A few grams of the material to be investigated are extracted with warm water containing 1% HCl or acetic acid. The

[1] Since free acetic acid is present in the filtrate after precipitation with lead acetate, one must add at least so much basic lead acetate that the liquid is neutral or weakly alkaline.

[2] If an acid liquid is added to a hot Fehling's solution, not freshly prepared, cuprous oxide is formed even in the absence of reducing substances (7). One must, therefore, either always prepare the Fehling's solution fresh or use for such tests an alkaline copper solution containing 3·5 gms. copper sulphate, 15·0 gms. of the purest glycerine, 25·0 Na-citrate and 20·0 (15%) NaOH in 100 c.cs.

filtrate is tested on a slide or in a test-tube with iodine potassium iodide and potassium mercuric iodide (general alkaloidal reagents). If there is no precipitate, no alkaloid is present.

Even a positive reaction with the so-called general alkaloidal reagents does not show the presence of alkaloids, since precipitates are obtained with other substances as well; for example, many tannins give precipitates with iodine potassium iodide, mucins and glucosides are precipitated with tannin, and carbohydrates give precipitates with silicotungstic acid and phosphomolybdic acid in the presence of hydrochloric acid.

d. The drug is shaken strongly with water and a little sodium carbonate which dissolves the saponin acids insoluble in water. If there is a high froth it may be caused among other things by vegetable mucins, tannins, proteins or saponins. The froth produced by the latter substances usually has a characteristic honeycomb structure and persists longer than that produced by other substances under similar conditions.[1]

The froth produced by proteins disappears when the liquid is boiled, and flocculent precipitates may be simultaneously formed.

e. A small portion of the material to be investigated is mixed with water and a few drops of chloroform in a test-tube or a small glass flask. The vessel is closed with a cork from the side of which is suspended a piece of paper soaked in picrate solution.[2] To a second test-tube, similarly prepared, some emulsin is added, and to a third (without chloroform) a little sulphuric acid. If the picrate paper is not coloured red after keeping a day, or even after the vessels 1 and 2 have been heated on a water-bath and the contents of vessel 3 heated to boiling (the cork of course being loosened), the presence of cyano-

[1] The same sort of froth as that produced by plants containing saponins is produced by Musa paradisica in which the property is due to the presence of potassium oleate.

[2] The picrate solution is a 1% solution of picric acid which has been mixed while warm with a 10% recrystallised sodium carbonate solution.

genetic substances is excluded.[1] If the paper in vessel 1 becomes coloured red when the substance is sprinkled with cold water, allowed to digest for several hours and then slightly warmed, but shows no colouration on warming after the addition of boiling water, then it may be assumed that the plant, besides containing a glucoside yielding HCN, contains also an enzyme capable of hydrolysing it.

These reactions serve only as preliminary indications. A more thorough investigation, especially when examining for glucosides, bitter substances and alkaloids, is possible by the process of Stas-Otto.

The Process of Stas-Otto

The method of investigation of Stas-Otto depends on the one hand upon the fact that most of the glucosides and bitter substances are soluble in alcohol and water and can be separated from aqueous solutions by shaking with ether or other solvents immiscible with water, and on the other hand upon the fact that alkaloid tartrates are likewise soluble in alcohol and water, but the alkaloids cannot generally be separated out from acid-aqueous solutions with ether and similar solvents as they pass over into these solvents only when they are liberated from their salts by alkalies. It should, however, be noted that a few alkaloids like colchicine and veratrine, with weak basic properties, can be shaken out with ether even in the presence of free acids, and that there are alkaloids, such as the phenol bases, which give compounds with fixed alkalies which are insoluble in ether.

The salts of the alkaloids must be decomposed with ammonia. Taking these facts into consideration, the Stas-Otto method is carried out in the following manner: The substance to be investigated (about 25–100 gms.) is boiled with about 2–5 times

[1] For further reactions of HCN and other plant products mentioned in this book, see L. Rosenthaler's *Nachweis organischer Verbindungen*, 2nd Edition, Stuttgart, 1923.

its weight of alcohol containing tartaric acid [1] for half an hour under a reflux condenser. The filtrate is freed from alcohol on the water-bath and the residue taken up first with a little, then with more water, stirred, heating on the water-bath when necessary, and the aqueous liquid filtered after cooling. If the liquid does not become clear it is evaporated to the consistency of an extract, or, when possible, to dryness. It is then taken up with alcohol and worked as before in order to get a clear aqueous filtrate. This is first of all shaken several times with ether in a separating funnel.[2] The liquid obtained by the first shaking (A_1) is kept separate when it is strongly coloured because the following ones (A_2) are likely to be less coloured.

A sample of the aqueous liquid should be tested with alkaloid reagents as on p. 21. If there is no precipitate the process can be discontinued at this point ; if there is a precipitate the liquid is made strongly alkaline with caustic soda and shaken repeatedly with ether without filtering off the precipitate that may be formed. The shakings thus obtained are combined together (B).

A sample of the liquid is acidified and again tested as before for alkaloids. If there is a precipitate the alkaline liquid is freed from ether by warming, ammonium chloride [3] is added in order to form ammonia, and it is shaken with chloroform or amyl-alcohol (C). If the aqueous solution after again acidifying still gives a precipitate with alkaloid reagents *quarternary ammonium bases* must be present, assuming, of course, that the extraction has been quantitative. These are

[1] No more tartaric acid should be used than is necessary to make the liquid slightly acid. After boiling one should see that the liquid is still acid. If this is not the case, the boiling should be repeated after the addition of a fresh quantity of acid.

[2] To avoid an emulsion, one should never shake the separating funnel backwards and forwards but give it a ∞-like motion. If the ether is emulsified in spite of this, a little alcohol should be added. The liquids often separate out on slight warming or when allowed to flow through a moist filter.

[3] Instead of this one can also neutralise with HCl and make the liquid alkaline with ammonia.

best precipitated from the acidified solution with alkaloid reagents and worked further as on p. 45.

The liquids *A*, *B* and *C* are dried with anhydrous sodium sulphate and filtered, and the solvents are distilled off until about 5 ccs. are left. If nothing separates out the residual liquids are poured into watch glasses or crystallising dishes. *A* and *B* are allowed to evaporate completely in the air and *C* on the water bath. If there is a residue, which is almost always the case, or if precipitates are obtained by concentration of the liquids, the precipitate and residue *A* must be tested for glucosides, alkaloids, bitter substances and similar things, and the precipitates and residues [1] *B* and *C* for alkaloids.

For this purpose the residue *A* is first tested by the *general carbohydrate reactions*: To the substance, dissolved in a little water or alcohol, a few drops of a 20% solution of α-naphthol in alcohol are added, and a layer of concentrated sulphuric acid run in below the solution. If glucosides (or carbohydrates) are present, a blue or violet ring is formed; on shaking the whole fluid becomes similarly coloured. If thymol is used instead of α-naphthol a red colour is obtained.

If these colourations do not appear, glucosides (and carbohydrates) are absent. If there is a colouration the residue should be purified from traces of carbohydrates that may be present by dissolving it in absolute ether,[2] anhydrous acetic ether or petroleum ether, and (after filtering if necessary) evaporating the solution. The residue is tested once more with the general carbohydrate reactions, and if these tests are still positive it is heated with HCl in order to split up the glucoside.[3] If a glucoside is actually present at least two

[1] In residue *A* one may also find neutral and acid substances which may be shaken out with ether. If it reacts acid one should never omit to test for tartaric acid (see p. 124), since this can also go over into ether.

[2] *Absolute ether* is obtained by first shaking the commercial ether with water to free it from alcohol, the water dissolved in the ether is decomposed by sodium, and, lastly, the ether is distilled over sodium.

[3] Since many glucosides are not hydrolysed by heating at the usual pressure, one must repeat the experiment in negative cases in a pressure bottle or sealed glass tube at increased temperatures.

substances must be formed by hydrolysis, a reducing sugar and a non-sugar (aglucone), the latter often falling in a flocculent mass if, of course, it is insoluble in water. The sugar is first tested for by heating the liquid (which has been filtered if necessary and then made alkaline) with an alkaline solution of copper. The liquid is also tested by the osazone reaction which is applied after filtering and neutralising. 0·5 gm. of phenyl-hydrazine hydrochloride and 0·75 gm. of sodium acetate are dissolved in the cold liquid, filtered if necessary, and warmed on a boiling water bath for half an hour. A watch should be kept for the formation of a precipitate during the heating ; the liquid should then be allowed to cool with the water bath and the precipitate examined under the microscope. The osazones form yellow crystals of different shapes (see also the section on carbohydrates).

The residue *A* may also be examined according to the process of Bourquelot or of Bridel, by which glucosides hydrolysable by enzymes can be detected in the presence of carbohydrates such as cane-sugar, which are hydrolysed by saccharase (invertin). The residue is dissolved in 50 to 60 ccs. of thymol water, divided into two equal portions and worked as described on p. 27.

The residue is also tested for alkaloids. A little of it is dissolved in very dilute HCl, the solution is filtered if necessary, and one drop placed upon each of several glass slides. A drop of an alkaloid reagent (potassium mercuric iodide, iodine potassium iodide, tannic acid, picric acid, silico-tungstic acid, see also p. 21) is put by the side of each and the two drops are brought into contact with a glass rod. If an alkaloid is present a turbidity will be observed at the point of contact. It must, however, be remembered that a precipitate may not be formed even when a base is present. For example, caffeine is not precipitated by potassium mercuric iodide. Nevertheless, precipitates can always be obtained by a suitable choice of reagents. On the other hand, there are non-alkaloidal

substances which give precipitates with some of these reagents. Thus, a few glucosides and carbohydrates are precipitated by tannin. One should, therefore, test if possible for nitrogen by Lassaigne's method (see p. 17) when the reaction for alkaloids is positive.

If the residue *A* contains neither alkaloids nor glucosides it should be carefully examined for its taste. If the taste is bitter one may be dealing with a bitter substance.

The residues *B* and *C* are examined in the same way as *A* for alkaloids.

It may be mentioned here that in many plants basic substances may occur which cannot be considered as alkaloids in the narrower sense but which give precipitates with alkaloid reagents. Among others betaine and choline are widely distributed. *Betaine* is characterised by a difficultly soluble gold double salt, it gives a blue colouration with potassium ferricyanide and ferric-chloride, and reacts neutral ; *choline*, which reacts alkaline (cf. p. 168), gives a precipitate in alcoholic solution with an alcoholic solution of corrosive sublimate. Both substances produce tri-methylamine on heating with caustic potash, and give *Florence's Reaction* (like a few other similar compounds) (*8*). A few drops of a solution of the substance, evaporated on a slide, and mixed with a strong solution of iodine in potassium iodide, show crystals, which when seen under the microscope appear to grow and disappear again.

The method of Stas-Otto is not without its drawbacks. First of all, ether is not a satisfactory extracting fluid for many glucosides and alkaloids and can be replaced with advantage by chloroform. Since the latter tends during shaking to form more emulsion than ether, the best thing to do, when extracting with chloroform, is to use the *perforation method* (which can also be used in the case of ether [1]). The *perforator*

[1] Instead of shaking or perforation the *Quick Extraction Apparatus* of Appuhn-Brügmann-Nielsen (obtainable from Emil Dittmar and Vierth, Hamburg) can be used, as it is specially suited for extracting substances

is fixed by means of a bored cork in the mouth of a small flask, containing about 40 to 50 gms. chloroform ; some chloroform is first put in the perforator, and over this the aqueous liquid to be extracted. A reflux condenser is fitted on the top of the perforator. The small flask is heated on the water bath so that about 30 or 40 drops fall into the perforator per minute. The perforation should last for at least 2 hours.

A further disadvantage of the Stas-Otto process lies in the fact that there are substances which decompose on boiling with acids and also on treatment with alkalies in the cold. The greatest drawback of the method, however, is that a number of glucosides and bitter substances are partially insoluble in water and alcohol and therefore do not generally pass over into the liquids used for extraction.

Detection of Cane Sugar and Glucosides according to Bourquelot

Ninety to ninety-five per cent. alcohol, mixed with a few gms. of calcium carbonate, is heated until it boils in a fairly large flask on a water bath ; as soon as it begins to boil the plant material for investigation (250 gms.), cut into small pieces, is slowly introduced into the alcohol in such a way that the boiling is not interrupted. When all has been added the boiling is continued under a reflux condenser for 30 minutes more. The enzymes are destroyed by this treatment and the glucosides are extracted.

The extract is poured out, the residue is crushed as finely as possible and boiled again with alcohol. The combined alcoholic liquids are filtered and evaporated to dryness *in vacuo*. The residue is dissolved in water, made up to 250 ccs.,[1] filtered and treated with a little toluene as antiseptic.

easily soluble in water from larger quantities of liquids. For an extraction apparatus for larger quantities of liquids see also H. Franzen : *Zeitschr. f. physiol. Chemie*, 129, 307, 1923.

[1] With less material correspondingly less water is used.

The solution is divided into one part of 50 ccs. (*A*), which serves for comparison, and another part of 200 ccs. (*B*). Both are placed in small flasks provided with corks. One gram of yeast powder [1] is added to solution *B*, and both the flasks are kept in an incubator at 30°–35° C.

After two days 20 ccs. of the liquid are taken from each flask, 4 ccs. of basic lead acetate added,[2] the mixture filtered and the filtrate tested polarimetrically in a 2-decimetre tube. If cane sugar is present the liquid *B* will show a deviation towards the left as compared with solution *A*. In order to ensure that one is really dealing with cane sugar one should proceed in the following way : The amount of reducing sugar in each liquid is determined, and, from the difference, the amount of reducing sugar produced by the action of saccharase. Since this sugar may be considered as invert sugar the corresponding amount of cane sugar should be first calculated, and from this the change of rotation which would be caused by the hydrolysis of this amount of cane sugar. The value obtained by calculation must be equal to the observed rotation. If these two values are different one may be dealing with another sugar, hydrolysable by saccharase (raffinose, gentianose, stachyose), or an α-glucoside. If the observations are repeated daily until two successive experiments give the same value the cane sugar can be thereby estimated quantitatively.

To test for glucosides the liquid *B* is used after all the cane sugar has been inverted. It is first of all heated for 10 minutes at 100° C. by putting the stoppered flask over a boiling-water

[1] The yeast powder is prepared from fresh baker's yeast. This is first stirred with a little sterile water and rapidly filtered by suction. The mass is then mixed with 8–10 times its weight of 95% alcohol. After 12–15 hours the mass is filtered by suction on a Buchner funnel, using a pump, washed with a little alcohol and then with ether, and finally dried in a drying oven at 30–35° C. The dry product is protected against moisture and preserved in a well-stoppered flask. If a saccharase solution is to be prepared from this, 1 gm. is rubbed with 100 ccs. of thymol-water and filtered.

[2] For drugs containing large quantities of tannins more basic lead acetate should be used.

bath, then cooled, and 0·5 gm. of emulsin [1] added for every 100 ccs. of solution. The further investigation is done exactly in the same way as described above for cane sugar.

Since nearly all the glucosides, hydrolysable by emulsin, are laevorotatory and yield glucose on hydrolysis (*Bourquelot's Rule*)[2], one can conclude that a glucoside is present whenever the action of emulsin produces an increase in the reducing sugars and a change in rotation towards the dextro side (or a diminution of laevorotation).

From the change in rotation and the amount of sugar one can calculate the *Index of Enzymolytic Reduction* (*Reduction Index*), *i.e.* the ratio of the sugar produced in 100 ccs., calculated as glucose in milligrams, to the change of rotation in degrees (observed in a 2 decimetre tube). Every glucoside hydrolysable by emulsin is thus distinguished by a characteristic reduction index.

As an example for calculation the following will serve, which is taken from the work of M. Bridel and M. Braecke (*12*) on the seeds of Rhinantus Crista Galli :

	Rotation.	Amount of reducing sugar.
Initial - - - -	−8° 2′	0·511 gm.
After saccharase - -	−12° 7′	3·025 gms.
After emulsin - -	+1° 2′	4·939 gms.

[1] *Emulsin* can be prepared in the following way (method of Robiquet-Herissey, with modifications of Saint-Stéban [*11*] : 1 Kg. of sweet almonds is poured into boiling water in portions of 250 gms., kept there for one minute and the water then replaced by cold water. The shells can then be easily peeled off. The peeled almonds are cut as fine as possible and then macerated for 48 hours with 2 litres of a mixture of equal parts of water and chloroform water. The liquid is pressed out and treated with acetic acid (about 8 ccs.) in order to precipitate the casein. After 3–4 hours it is filtered and the filtrate mixed with thrice the volume of 95% alcohol. After 12 hours the supernatent liquid is decanted off, the precipitate filtered with a Buchner funnel, and washed first with alcohol and then with ether. It is dried *in vacuo* over sulphuric acid and then powdered. For other methods of preparation see the section on Enzymes.

[2] There are exceptions to this rule. For example, α-ethyl-l-arabinoside, which is hydrolysed by emulsin, is dextrorotatory. The rotation, however, becomes less on hydrolysis (M. Bridel and C. Béguin [*10*]).

Trans. Note.—Also gynocardin, which is hydrolysable by emulsin, is dextrorotatory (F. B. Power, *Trans. Chem. Soc.* 1905, p. 345).

By the action of saccharase the laevorotation has increased by 4° 5′ and the quantity of reducing sugar has increased by 2·514 gms. On the assumption that this is invert sugar it corresponds to 2·388 gms. of cane sugar. If we calculate from this the decrease in rotation which would be expected theoretically, using the formula: $\alpha = \frac{[\alpha] . l . c}{100}$, where $l = 2$, the specific rotation $[\alpha]$ for cane sugar = 66·5°, and for invert sugar = $-19{\cdot}82° - 0{\cdot}04\,p$, we find a change of 3·17° is expected from the disappearance of cane sugar and 0·99° due to the formation of invert sugar. The total change expected, therefore, = 3·17° + 0·99° = 4·16° = 4° 10′, while the observed value was 4° 5′. The calculated reduction index becomes $\frac{2514}{4{\cdot}08} = 616$, while the theoretical value for cane sugar = 601.

If the reduction index for the hydrolysis with emulsin is calculated it is found to be $\frac{1914}{13{\cdot}33} = 143$, which agrees well with the theoretical value for aucubin. The content of aucubin in the seeds can also be calculated, and is found to be 3·41%.

The reduction indices for a few of the widely distributed glucosides are given as follows:

Amygdalin	490	Picein	261
Arbutin	700	Sambunigrin	281
Meliatin	250	Aucubin	about 140
Methyl-arbutin	326	Coniferin	278
Amygdonitrile glucoside	517	Salicin	321
Gentiopicrin	111	Verbenalin	19

The process of Bourquelot naturally fails in the case of all glucosides which are not hydrolysed by emulsin. It also leads to difficulties in cases where the glucoside (*e.g.* verbenalin) or he aglucone (*e.g.* that of arbutin) acts as a reducing agent.

It may also happen that the aglucone renders the emulsin inactive and thus prevents the complete hydrolysis of the glucoside. In such a case emulsin should be added again, and

the process repeated until there is no more change of rotation. The possibility must also be reckoned with that there may be glucosides present which are split up by saccharase.

And, finally, it must be borne in mind that all the estimations of sugar, which depend upon the reduction of alkaline solutions of copper, may be defective in the presence of substances containing nitrogen (*12a*).

A number of glucosides (primveroside, rutinoside, rhamninoside), which are not split up by emulsin, are hydrolysed by the enzyme rhamnodiastase.[1] M. Bridel and C. Charaux (*13*) have based upon this *a biochemical method for the identification of glucosides* which is analogous to Bourquelot's process. The glucoside is treated as in the other case first with saccharase and then with rhamnodiastase (0·05–0·1 gm. for 100 ccs. of liquid) instead of emulsin. Its action is comparatively more rapid and is usually complete in 48 hours. For polarimetric estimation the liquid must be thoroughly purified with basic lead acetate.

The Lead Method

If the preliminary tests with lead acetate and basic lead acetate (see p. 20) give a positive result a portion of the substance to be investigated should be examined by the lead method (specially worked out by Rochleder). The material is boiled with water (if necessary first extracting with ether or petroleum ether), and the filtered, clear aqueous liquid (best boiling hot) is mixed with a solution of lead acetate in slight excess. The lead precipitate (*A*) thus obtained is washed

[1] To prepare *rhamnodiastase* (*14*), the crushed fresh seeds of Rhamnus utilis Decaisne are first extracted thoroughly with ether and the residue macerated for 12 hours with four times its volume of thymol-water. The mass is pressed and filtered and the filtrate treated with four times its volume of 95% alcohol. The precipitate is filtered, washed with alcohol and ether, and dried over sulphuric acid or in a drying oven at 30° C.

The rhamnodiastase can also be prepared in the same manner as emulsin (see p. 29, note 1).

Or, finally, one can use even the white kernels of the seeds, which have been powdered and extracted with ether. The residue is treated further as in the first method.

first with a solution of lead acetate and then with water [1] until the washings are no longer acid. The filtrate (including the washings, concentrated by evaporation) is precipitated with basic lead acetate, the details of procedure being the same as with lead acetate, and the precipitate (*B*) is separated from the liquid (*B*) in the same way as with *A*. The precipitates *A* and *B*, as well as the liquid *B*, must be further examined.

It should next be determined whether the precipitate (*A*) is soluble in cold or boiling alcohol, even if only partially soluble. If any dissolves, which is known by the residue left on evaporating a small portion of the alcoholic liquid over a watch-glass, the liquid ($A\alpha$) is freed, if necessary, from lead [2] by H_2S,[3] the filtrate is concentrated and finally allowed to evaporate *in vacuo* over H_2SO_4. The portion of the precipitate *A*, not dissolved by boiling alcohol, is treated with 10% acetic acid. If the precipitate (*A*) does not dissolve in it completely one can ascertain whether any of it at all has dissolved by adding basic lead acetate to the liquid.[4] If there is a precipitate the solution is completely precipitated with basic lead acetate, the precipitate ($A\beta$) is washed, suspended in water and treated with H_2S. The filtrate is concentrated [5] and finally allowed to evaporate *in vacuo* over H_2SO_4. The portion of the precipitate *A*, not dissolved by treatment with alcohol and acetic acid, is also treated in the same way (*i.e.* suspended in water and decomposed with H_2S).

[1] Since these lead precipitates very often choke the filter quickly, they are best shaken with the washings in a glass cylinder, allowed to settle, the supernatant liquid decanted off and the residue, already washed, then placed upon the filter.

[2] All the lead sulphide formed during the investigation should be washed and separately preserved for further examination.

[3] Instead of removing Pb by H_2S it is occasionally advisable to precipitate Pb by dilute sulphuric acid, phosphoric acid or their sodium-salts.

[4] Lead carbonate, which might be present in the precipitate if the water used contained CO_2, is not here taken into consideration.

[5] To avoid the separation of sulphur, frequently produced by the evaporation of liquids containing H_2S, and which is difficult to remove by filtration owing to its fine state of division, one may displace the H_2S by a current of CO_2 before the evaporation.

With the precipitate (*B*) [1] the procedure is the same as with precipitate (*A*), but the treatment with acetic acid is omitted since the precipitate obtained with basic lead acetate is wholly soluble in acetic acid.

The liquid *B* which, besides lead and acetic acid from the reagents employed, may also contain glucosides, bitter substances, alkaloids, sugars, salts and the like, is freed from Pb, PbS and H_2S in the usual way [2] and divided into three parts for further treatment. The *first* portion is treated with sodium carbonate until it reacts faintly acid and then treated further according to the method of Stas-Otto (see p. 22). The *second* portion is concentrated and allowed to evaporate *in vacuo* over sulphuric acid. If crystals come out they are separated and the mother-liquid is again concentrated, filtered from any crystals that may be formed and then evaporated to dryness. The residue is taken up with alcohol, and the alcoholic solution is treated with ether in order to precipitate any substances that may be insoluble in ether. It may in this way be divided again into three parts, a portion insoluble in alcohol, another insoluble in ether and alcohol, and a third soluble in these. In order to ascertain whether decompositions take place by this process through the free acetic acid the *third* portion is neutralised with sodium carbonate before the evaporation and treated in the same way as the second portion, taking however, into consideration the fact that sodium acetate is soluble in water and alcohol.

There yet remains to examine the lead sulphide obtained during the course of the investigation. Lead sulphide in its nascent state possesses the property of bringing down all sorts

[1] Before decomposing the lead precipitate with H_2S it is advisable to treat it with CO_2 in order to decompose the compounds with carbohydrates or inosite (Franzen and Helwert). The treatment of the precipitate with CO_2 and H_2S is simplified by the use of a shaking machine. See H. Franzen, *Zeitsch. Physiol. Chemie*, 122, 86, 1922.

[2] Before passing sulphuretted hydrogen one may test a portion of the liquid to see whether any precipitate containing organic constituents is obtained by the addition of ammonia, since a few carbohydrates may be precipitated in this manner.

of things, especially colouring matter and substances which produce turbidity, and one of the advantages of the lead method depends upon this. The lead sulphide is treated successively with boiling water, boiling alcohol and ammonia, and each liquid is evaporated in order to ascertain whether anything has gone into solution. Finally, the lead sulphide so treated is oxidised with hydrogen peroxide (*15*), and the lead sulphate formed is boiled with water and then with alcohol. The behaviour of a small amount of the lead sulphide with the reagents mentioned should first be tested of course before taking up the whole amount for work.

In this way a large number of crystalline substances and amorphous residues are obtained which have to be identified. This is simplified by taking into consideration the circumstances in which they were obtained. For example, the precipitate *A* may contain vegetable acids, tannins, mucins and glucosides. The substance obtained from the precipitate *A* should therefore be tested with Fehling's solution in the same way as described before (see p. 20), as also the substances obtained from the liquid and precipitate *B*. From the precipitate and liquid *B* one may obtain, among other things, basic substances, as well as the sugars. (These only occur in the precipitate when they are soluble with difficulty in water in the free state.)

The disadvantages of the lead method, which are predominant and seldom avoidable, are the use of H_2S (for the complete removal of Pb) which is not without action upon many organic substances, and the formation of free acetic acid (obtained during the precipitation with lead acetate and during the final removal of Pb) which may also produce decompositions. If the acid is neutralised the isolation of other substances [1] is hindered by its salts, which are soluble in water and alcohol.

[1] The separation is also often incomplete. If, for instance, the precipitate with lead acetate is soluble in acetic acid then the precipitation is incomplete, since acetic acid is set free by the double decomposition. If basic lead acetate is then added the acetic acid is neutralised, and the portion of the lead acetate precipitate held in solution by it goes over to the basic lead acetate precipitate.

If precipitates are only obtained in the extract with one of the lead salts (be it neutral or basic lead acetate), one can often avoid the introduction of acetic acid by using *freshly precipitated lead hydroxide* (or, possibly, lead carbonate) as the precipitating agent. The precipitate and filtrate are treated further as the precipitate and liquid *B*.

Apart from the systematic use of the lead compounds [1] described here they are often employed in phytochemical analysis for the clarification and decolourisation of liquids.

In many cases it may be advantageous to carry out the lead method in alcoholic solution.

The salts, hydroxide and carbonate of copper act in the same way as the lead compounds. Separations may be effected by their means in the same way. One may, for example, precipitate first the tannins and mucins with the carbonate and then the other compounds with the hydroxide.

The General Method

The principle of the method consists in the treatment of the coarsely powdered material with a series of different solvents in succession. The residues obtained by evaporation of the extracts are further examined either on the same principle or by the lead method. It is often simpler to shake the extracts with suitable solvents than to evaporate them and take up the residues over again. In general 250 gms. of the material suffice for such a preliminary investigation. In order to determine whether the action of hot solvents causes decompositions the same amount of material may be extracted with the same solvent simultaneously in an extraction apparatus with the aid of heat and in a percolator in the cold. As a rule each solvent should be allowed to act until no further substance is extracted (see p. 12) ; one may proceed with a second solvent before complete exhaustion with the first only when the action of the

[1] In place of lead hydroxide one may occasionally use magnesium oxide or freshly precipitated aluminium hydroxide.

first solvent is insignificant. In order to secure a uniform extraction of the material it is taken out of the apparatus from time to time, mixed thoroughly and replaced in the apparatus. When changing the solvent the material should be freed from the previous solvent by evaporation (except from extraction 6 onwards). Sufficient information concerning the substances present in the drug will in most cases be secured by the following process.[1]

I. Solvent: *Petroleum ether*, B.P. 35°–40°,[2] which can easily be prepared by fractional distillation of the commercial article. The following substances may be dissolved by petroleum ether: fats and fatty oils, waxes,[3] essential oils, colouring matters, phytosterols and less often alkaloids (present in the free state), glucosides and resinous substances. The alkaloid is extracted from the petroleum ether by shaking with acidified water. If this is made alkaline and again shaken with petroleum ether the alkaloid in the free state dissolves in the petroleum ether and can be obtained from it by evaporation. In case a water-soluble glucoside is present (which is not very probable) this should be looked for in the same residue. The same holds good for other substances soluble in water and petroleum ether.

The petroleum ether solution remaining after shaking with acidified water may be freed from any acid that dissolves in it by washing with water, and the petrol ether is then distilled off. The residue is taken up with boiling 90% alcohol in which all the substances still present, with the exception of fats and fatty oils, will dissolve.[4] Waxes and phytosterols

[1] Tr. note. The whole process with organic solvents (petroleum ether, ether, chloroform and absolute alcohol) can best be carried out in the extractor devised by Wester (see Appendix A).

[2] Tr. note. For tropical climates one may use up to 60° C.

[3] Also lecithin, see p. 97.

[4] According to the particular nature of the oil or fat a larger or smaller portion of this may also be dissolved by the hot alcohol. In such cases one may again concentrate the alcohol and try to effect a separation of the remaining substance with dilute alcohol, ether, benzene, etc.

separate out along with some of the dissolved fat on cooling the alcohol and may be identified as shown on pp. 78 and 92. One should then see if anything can be separated out with ether. If not, the solvent is evaporated off and the residue is treated with ether, methyl alcohol, benzene and similar solvents in order to effect a separation of the constituents still present. In most cases this will be possible; if not, the essential oil is distilled off in steam and the resin is removed, if possible, by taking up with KOH from the glucoside, the presence of which has already been confirmed as described on p. 21 and p. 24. Instead of this method of separation one may try to take up the residue in alcohol and precipitate fractionally with water. In most cases petroleum ether is not found to dissolve so many substances as have been assumed in this example, and the separations should, therefore, be easier to accomplish.

II. Solvent: *Absolute ether.* If the substances soluble in petroleum ether have been fully extracted, ether will eventually dissolve out other glucosides and alkaloids, other resinous components and colouring matters, ether-soluble acids like gallic acid and indifferent bodies. The ether is evaporated off and the residue tested for glucosides and alkaloids. The residue is extracted in succession with water,[1] acidified water (in case an alkaloid is present), alcohol, carbon disulphide and other solvents. If resinous substances have been extracted one should again try to remove them with KOH from the ethereal solution or to precipitate them from the alcoholic solution with water.

III. Solvent: *Chloroform.* This will dissolve essentially the same substances as ether, and is moreover a good solvent for caoutchouc-like substances.

IV. Solvent: *Absolute alcohol.* The substance is taken out of the extraction apparatus, boiled with alcohol and filtered through a steam-heated funnel. On cooling the

[1] The aqueous solution is tested with ferric chloride.

alcohol[1] precipitates of salts, saponins or other glucosides of similar behaviour and sugars are obtained. Salts are provisionally tested after converting into ash on a platinum foil, and glucosides and sugars are tested as on p. 20 and p. 24.

The filtrate is freed from most of the alcohol by distillation, filtered from any portion that may have separated out, and the filtrate is precipitated with ether. The precipitate may contain among other things (besides those mentioned before) tannins or salts of alkaloids. It is dissolved in water and tested for these substances in the usual way. In the substances not dissolved by water one should test specially for *phlobaphenes* (see p. 119), decomposition products of tannins, which are soluble in alkalies and can be precipitated from these solutions by acids.

The substances found in aqueous solution should be separated by the lead method (see p. 31).

The ether-alcoholic solution is concentrated and the residue is tested for tannins, alkaloids, glucosides, etc., and the lead method is followed in cases where a separation cannot be effected by solvents.[2]

V. Solvent: *70 per cent. alcohol.* The material is boiled and filtered hot. Any precipitate that may be formed on cooling is filtered off and the alcohol distilled from the filtrate, from which a precipitate may possibly be obtained again on cooling. The substance in solution, which is approximately the same as that which goes over into absolute alcohol, can be isolated by the lead method.

VI. The sixth solvent is *cold distilled water*.[3]

In water one may find some bitter substances and glucosides which may not have been dissolved by the solvents used hitherto: and also substances not dissolved by alcohol, such as sugars, salts, gums, mucins and proteins, the latter only

[1] As it may contain plant acids in solution (see p. 119) the reaction should be observed.

[2] On taking up with water the resins may again separate out.

[3] To avoid fermentations one may use water saturated with chloroform.

provided that they have not been rendered insoluble by the previous treatments.

For proteins one should test by the *general protein reactions :*

(1) Heating. In the presence of albumins a precipitate is formed which is insoluble in acetic acid and in nitric acid.

(2) Precipitates are obtained with alkaloidal reagents ; those with albumoses may be redissolved by an excess of the precipitating agent.

(3) The Biuret reaction. Mix with caustic potash and a few drops of cupric sulphate solution. Blue or bluish-violet colour shows the presence of albumin ; red, that of albumoses.

(4) In most cases lead sulphide is formed by boiling with a lead salt and KOH.

(5) When warmed with strong nitric acid proteins are coloured yellow, changing to orange on treatment with ammonia.

(6) Some of the proteins give a red colour on warming with Millon's Reagent.

The aqueous solution, whether proteins are present or not, is mixed with an equal volume of alcohol. Mucins, proteins and some salts are precipitated thereby. One should redissolve the precipitate in water and try to separate the salts from mucins and proteins by dialysis, and to separate the proteins from mucins by heating with a little acetic acid or by salting out. In cases where this is not possible the lead method should be followed, since the proteins are precipitated by lead acetate and the mucins are often precipitated first with basic lead acetate. It is often impossible to free the mucins completely from nitrogenous constituents. From the aqueous-alcoholic solution the alcohol is distilled off and the lead method followed.

VII. *Extract with boiling water.* The starches become pasty thereby. Mucins, xylans, inulin, pectins, glycogen, lichenin, etc., which are soluble with difficulty, go into solution. The inulin can be crystalised out by evaporation. The other

substances are precipitated in the amorphous state by alcohol. For their properties see pp. 150 *et seq.*

VIII. Solvent: *Cold 1% hydrochloric acid* with which the substance is digested for several days with frequent shaking. This dissolves the alkaloids which may have so far escaped extraction, and also salts of vegetable acids which are soluble with difficulty, like calcium tartrate and calcium oxalate and proteins. The latter are tested for as before, and, if present, the solution is salted out with ammonium sulphate. To test for vegetable acids the liquid [1] is boiled with sodium carbonate and examined further as described on pp. 119 *et seq.*

IX. On *heating with* 5% NaOH the pentosans and other membrane-substances, besides proteins and hemi-celluloses, are extracted. For further information see pp. 152 *et seq.* Phlobaphenes (see p. 119) may also go into solution.

X. *Heating with dilute acids* eventually brings even the carbohydrates into solution. Cellulose and a portion of the lignin remain behind. The residue is tested first for lignin, which becomes yellow with aniline sulphate and sulphuric acid, green or blue with phenol or thymol and HCl, and cherry-red with phloroglucin and hydrochloric acid.

XI. Lignin can be separated from cellulose in several ways; it is dissolved by a mixture of potassium chlorate and nitric acid, and also by sulphite-liquor,[2] while cellulose remains behind. On the other hand, only cellulose goes into solution (see p. 154) when the residue is treated with ammoniacal copper oxide. From the solution the cellulose is precipitated by hydrochloric acid.

Cellulose can also be separated from lignin by means of 41% HCl, which dissolves the cellulose, gradually transforming it to dextrose (*16*).

[1] If starches or other carbohydrates, soluble in HCl but soluble with difficulty in water, were present, dextrin or reducing sugars should be present in the concentrated liquid.

[2] *Sulphite liquor* is obtained by passing sulphur dioxide into a mixture of 50 gms. calcium carbonate and 1500 gms. water, until all the $CaCO_3$ is dissolved. It does not always dissolve the lignins completely.

The above general method can be altered as follows according to J. Zellner (*16a*) :

The air-dried, finely powdered material is boiled with 95% alcohol ; the extract is treated with warm water, and the substance insoluble in it filtered off and dried *in vacuo*. This is then extracted completely with petroleum ether (I) and ether (II), whereupon a residue (III) is obtained. The water-soluble portion of the alcoholic extract is concentrated *in vacuo* and shaken out with ether and chloroform (IV) ; the aqueous extract remaining (V) can be worked out by the lead method (see p. 31). The material fully extracted with alcohol is finally extracted with water (VI) and tested as above.

SECTION II: SPECIAL

I

ALKALOIDS

If the preliminary examination has shown that the isolated alkaloid is volatile the drug may be directly submitted to distillation after adding a non-volatile alkali to the pulverised material, or one of the extracts (made alkaline) may be used for distillation. The extracts may be obtained by the general methods of preparation described below, according to which the volatile alkaloids can also be obtained without distillation, since most of them are not volatile at 100° (without steam).

The isolation of alkaloids involves three stages : 1. Extraction, 2. Preparation of the crude alkaloid, 3. Purification of the crude alkaloid and eventually the separation of several akaloids from one another. In most cases the operations (2) and (3) cannot be strictly separated from one another.

For extraction three methods come into consideration :

(*a*) Extraction with neutral solvents. In a few cases the alkaloids or their salts may be extracted simply by ether, alcohol or water.

(*b*) Extraction with acid solvents. The acids may be dissolved to the extent or 1 or 2 per cent. in water or alcohol. Mineral acids are mostly used, usually hydrochloric acid or sulphuric acid, seldom organic acids ; the latter are used only when it is to be feared that the alkaloid may be decomposed by mineral acids.

According to a suggestion by S. Ghosh (*a*) and (*b*) can also be combined as follows. The material is extracted cold or hot with alcohol and the alcohol is removed, best *in vacuo*. The residue can either be dissolved in a little cold glacial acetic acid, and the solution poured with constant stirring into so much

water that a one per cent. solution of acetic acid is obtained, which keeps the alkaloid in solution ; or the residue may be taken up with 0·5–1% HCl or H_2SO_4 by thorough shaking. The alkaloid can then be set free by means of alkalies (see p. 44).

(*c*) Extraction in alkaline solution. One method is to mix the powdered drug with the alkali (using for the purpose weak alkalies like calcium or barium hydroxide in order to avoid decomposition), moisten the mixture of the drug and alkali, dry the mass and then extract with a suitable solvent. Such solvents are : hydrocarbons of the fatty series (petroleum ether, benzine, ligroin, paraffin oil) and some of the benzene series (benzene, toluene, xylene, etc.), alcohol, carbon tetrachloride, ether, acetone, etc.

Alternatively a solvent made alkaline may be used and alkali ammonia serves best for the purpose. Good results are obtained with ammoniacal alcohol and chloroform.[1] To prepare ammoniacal liquids the commercial aqueous ammonia may be used. The ammonia is liberated from it by heat, passed over quicklime for dehydration and then through the solvent.

An alternative method for removing acid and neutral impurities from the weakly acid medium before extracting in the presence of alkali is that patented by Stoll and used in the preparation of ergotamine from ergot. By moistening with a 5% *solution of aluminium sulphate* the basic groups in an amphoteric substance are saturated and the acids set free. If it is then extracted with ether and benzene the acids and neutral substances are removed. Alkali is added to liberate the base, which can then be extracted with the solvents mentioned.

By means of the latter methods it is possible in favourable cases to obtain a tolerably pure crude alkaloid by merely evaporating the solvent. In particular cases the alkaloid may

[1] In using chloroform it must be remembered that many alkaloids combine with chloroform or may be decomposed by chloroform as by alkalies.

also be obtained by shaking the aqueous liquid [1] (prepared by extraction methods (*a*) and (*b*)) with a suitable solvent, which will take up the alkaloid, and then evaporating off the solvent. The liquid, however, should never be evaporated to dryness, but the concentrated liquid should be allowed to evaporate slowly *in vacuo* to make the formation of crystals possible.

For the most part, however, the alkaloid must be precipitated with an alkali by using methods (*a*) and (*b*). For this purpose the alkali bicarbonates and carbonates may also be used in many cases, and also the hydroxides of the alkaline earths besides ammonia and the alkali hydroxides. It has already been remarked (p. 22) that a separation of several alkaloids present simultaneously may be attained by using these precipitating agents in succession, and also that a few alkaloids are soluble in caustic alkalies. The precipitated alkaloids are collected on a filter or suction-filter and freed from mother-liquor in the usual way.

If an alkaloid separates out with the alkalies one should try to shake it out with ether, chloroform, benzene, etc. In some cases (galegine), amyl-alcohol has proved to be a suitable solvent. From the solution thus obtained the alkaloid is obtained either by evaporating the solvent or by shaking out with dilute acids. The salt may either be prepared direct from the solutions or the base may be precipitated after concentration by means of alkalies.

It is often preferable to submit the liquids containing alkaloids to a purification before precipitating the alkaloids.

(α) As in the Stas-Otto method the material may be taken up repeatedly with water and alcohol.

(β) The liquid is decolorised with charcoal, best with animal charcoal which has been freshly ignited. This process cannot always be recommended since many alkaloids are adsorbed by

[1] If water has not been used for extraction the solvent is evaporated and the residue taken up with water.

animal charcoal to no inconsiderable extent. In all cases the charcoal must be extracted with acidified water and tested for the presence of alkaloids with alkaloid reagents.

(γ) The aqueous or alcoholic liquid should be treated with lead acetate or basic lead acetate. The excess of lead salts is removed by sulphuretted hydrogen, etc., as usual. Rochleder has already observed that in precipitating aqueous liquids with basic lead acetate the alkaloids which are soluble with difficulty in water may be found in the precipitate.

(δ) By shaking. Impurities can often be removed from the neutral or acid liquids by shaking with a suitable solvent (see above) without any appreciable loss of alkaloid. The liquid is made alkaline and again shaken with a solvent, the alkaloid going over to the second solvent and a large portion of the impurities remaining behind in the aqueous liquid. From the second solvent the alkaloid may be transferred into acidified water by shaking; again made alkaline, shaken with the second solvent, and these operations repeated until a sufficient degree of purity is attained. If the alkaloid is colourless the gradual decolouration of the liquid marks the advent of purity. The shaking should be repeated in every operation until the liquid shaken contains no more alkaloid, this being ascertained by an alkaloid reagent. With aqueous liquids this can be ascertained directly, but with other solvents a small portion should be evaporated on the water-bath and taken up with acidified water (see p. 21).

(ε) A very rapid and thorough purification is frequently attained by precipitating an alkaloid from its solution with an alkaloid reagent and decomposing the precipitate to regain the alkaloid. The alkaloid set free can be obtained pure by shaking, and so on. The shaking offers the advantage in this case that non-alkaloidal matter, such as proteins, which are precipitated by many alkaloid reagents, are removed from the free alkaloid, an object which can also be attained usually by simply taking up the residue with a non-aqueous solvent.

Among the precipitating agents may be mentioned :

(*a*) *Tannic acid*—The precipitate, rubbed fine with water or alcohol, is decomposed by warming with freshly precipitated lead hydroxide, or, similarly, with freshly precipitated lead carbonate. Magnesium oxide or zinc oxide may also be used for the same purpose. The alkaloid set free is either shaken out of the aqueous solution or obtained by evaporation after filtration, and so on.

(*b*) *Kraut's potassium bismuth iodide solution*[1]—Precipitation is carried out in a solution acidified with sulphuric acid. The moist precipitate is rubbed with so much silver carbonate that the red colour of the mixture disappears and the filtrate no longer gives the iodine reaction. Any silver which may be present in the solution is removed by H_2S.

Alternatively, the precipitate may be decomposed by boiling with excess of barium carbonate and water, and filtered and washed.[2] The combined filtrates are evaporated to the consistency of a syrup, treated with excess of baryta water and shaken out with ether.

The aqueous liquid is neutralised with dilute sulphuric acid and then treated with so much silver sulphate, or, on account of its difficult solubility, with so much sulphuric acid and silver carbonate that a filtered sample does not show any reaction for iodine. The precipitate is filtered and washed, the filtrate freed from silver by H_2S, freed from H_2SO_4 by careful pre-

[1] This reagent is prepared (*17*) by dissolving 80 grams of basic bismuth nitrate in 200 gms. of HNO_3 of sp. gr. 1·18 and pouring this solution into a concentrated aqueous solution of 272 gms. of potassium iodide. When the potassium nitrate crystallises out the liquid is diluted to one litre.

Instead of Kraut's reagent one may also use bismuth barium iodide, or bismuth ammonium iodide (C. Neuberg). To prepare the former, 118 gms. bismuth iodide are dissolved in a concentrated solution of 65 gms. barium iodide, and finally diluted to 500 ccs. after the addition of a few drops of hydriodic acid and hydrochloric acid ; to prepare the ammonium compound 118 gms. of bismuth iodide are added to a solution of 43·5 gms. ammonium iodide in 200 ccs. of water and diluted to 500 ccs.

[2] It is a good policy to extract the residue with alcohol, etc.

cipitation with baryta, and then concentrated or worked up in some such suitable manner.

(*c*) *Phosphomolybdic acid*, *silico-tungstic acid* or *phosphotungstic acid*—The precipitates are decomposed by the hydroxides or carbonates of alkalies and alkaline earths, if necessay with the aid of heat.[1] The liquid is worked up by a suitable method such as extraction with ether, etc. If barium hydroxide is used the liquid is previously freed from excess of barium by a current of CO_2.

(*d*) *Potassium mercuric iodide*—The precipitate is similarly decomposed by the hydroxides or carbonates of alkalies and alkaline earths.

(*e*) *Platinum chloride* or *gold chloride*—From the precipitates, finely divided in water, the precious metals can be removed by H_2S or by alkali carbonates.

(*f*) *Tartaric acid*—In many cases the alkaloids may be precipitated out of aqueous or aqueous-alcoholic solutions as bitartrates. According to a patented process (*18*), the precipitate has been worked out as follows: The precipitate was washed with alcohol and taken up with water, whereby the cream of tartar remained undissolved. The solution was made normal with sulphuric acid and treated while stirring with portions of phosphotungstic acid until a sample of the filtrate showed no more turbidity with 10% aqueous phosphotungstic acid. The precipitate was decomposed by shaking with a solution of lead acetate, the solution freed from lead, again concentrated, and the alkaloid again precipitated out of an alcoholic solution of the residue by tartaric acid.

(*g*) *Anthraquinone-β-monosulphonic acid*—For example, α-lobeline is obtained by precipitating its alcoholic solution with an alcoholic solution of the reagent and decomposing the precipitated salt with ammonia.

[1] One should avoid heating, if possible, since many bases are decomposed thereby.

If the alkaloid obtained in this way is not sufficiently pure (it may be still somewhat coloured or it may not show any tendency to crystallise), it must be purified further. For this purpose it is either treated with animal charcoal or one of the methods of preparation described above may be repeated, either by shaking or by precipitating and decomposing the precipitates. One may also try to take up with different solvents, or try precipitation methods such as precipitating a solution in absolute alcohol with petroleum ether or water, etc.

A method often followed for purification consists in preparing salts (or double-salts), since these often crystallise better than the free bases, and then recovering the bases by decomposing them.

To prepare a salt, the base is neutralised with the acid in the presence of water or alcohol and the salt is recrystallised from a suitable solvent. From the aqueous solution one may often precipitate the salt with alcohol, or from the alcoholic solution with ether. To prepare one salt from another one may make use of double-decomposition; thus, the chloride may be obtained by treating the alkaloid-sulphate with barium chloride.

Gold and platinum double-salts (see above) often crystallise well.

Among the most difficult and the most interesting tasks in phytochemistry is that of ascertaining whether the alkaloidal component obtained from a plant consists of more than one alkaloid, and, if it does, of separating these alkaloids from one another. The separation may even be attained in course of the preparation, *e.g.* one alkaloid may be shaken out from the neutral liquid or the liquid made alkaline with a fixed alkali, while perhaps the second alkaloid would go over only from the liquid made alkaline with ammonia. Any different behaviour of the alkaloids with solvents may also be utilised for the separation. Several solvents in succession may be used for shaking during the course of the preparation, *e.g.* first petroleum ether, then ether, and, lastly, chloroform; or one may try the

action of different solvents upon the precipitated alkaloid. There is also the possibility of separation during the purification. The constituents of the mixture of alkaloids may behave differently with the physical or chemical agencies acting during the precipitation. When an alcoholic solution is precipitated with ether one component may possibly be soluble in ether-alcohol, or it may be precipitated by platinic chloride in aqueous solution while the accompanying substance is first precipitated by the addition of alcohol, and so on.

Fractional saturation with acids, depending upon the different basicity of alkaloids, is likewise a very suitable method of separation. The amount of acid necessary to neutralise the mixture of alkaloids should first be determined by trial, the mixture dissolved in a solvent immiscible with water, and then shaken, with a tenth part of the acid required to neutralise the alkaloids, ten times. From each fraction the alkaloid is set free and its properties ascertained.

For physico-chemical methods for the separation of alkaloids see J. M. Kolthoff, *Biochem. Zeitschr.* **162**, 289.

As an example of a complicated separation the method by which J. Gadamar and K. Winterfeld (*19*) worked out the secondary alkaloids of Chelidonium majus may be described.

The total mixture of bases was dissolved in a sufficient amount of warm alcohol and treated gradually with the calculated quantity of alcoholic picric acid with constant stirring, and after the addition of more alcohol the mixture was heated to moderate boiling on the water-bath. A portion of the picrate was precipitated thereby and another portion came out on cooling. The filtrate was concentrated slowly, and the portions solidifying out were separated into eight fractions according to their solubilities.

The decomposition and working out of the single fractions were done as follows. They were finely rubbed with water, made into a paste and transferred to a separating funnel ; after acidifying with HCl they were shaken with ether until the

picric acid went almost completely into the ether. The solution of the alkaloid in aqueous HCl was made alkaline with ammonia and again shaken with ether. The ether was distilled off, the residue acidified diluted with water, and again shaken out with ether after the addition of an excess of ammonia. These processes had to be repeated several times before the last traces of picric acid could be removed and the ethereal solution became colourless. From the concentrated ethereal solutions of the higher melting picrate fractions the alkaloid crystallised out on standing.

As the free bases obtained from the low-melting picrate fractions could not be crystallised they were converted into *perchlorates*. The solution of the bases in aqueous HCl was fractionally precipitated with perchloric acid. The single precipitates were kept aside for several weeks. They were then filtered by suction and dried between filter papers over H_2SO_4. Each fraction was finely powdered, dissolved in hot water and shaken exhaustively with ether after saturation with ammonia.

The bases so obtained could be further partly separated by treating the thiocyanates prepared from these with alcohol in which only a portion was easily soluble.

The quantitative estimation of alkaloids may be done by the *gravimetric* or volumetric method. The method adopted in the first case would depend upon the method of isolation and properties of the alkaloid.

In many cases one may proceed in the following way : the powdered material is transferred with the solvent, not miscible with water, and alkali (caustic alkali solution, ammonia, soda solution) added. After sufficient shaking a definite portion of the solution is poured out or filtered and shaken thoroughly with dilute HCl. The acid extract is again made alkaline, shaken thoroughly with a suitable solvent, the solution evaporated and the residue dried to a constant weight.

For the *volumetric method* the equivalent weight of the

alkaloid or alkaloidal mixture must be known. To carry out an estimation several methods may be adopted :

(*a*) The material is shaken thoroughly with an alkaloidal solvent not miscible with water (ether, chloroform, mixture of both) and then the alkali added. After a time, during which it should be frequently shaken, a definite portion of the liquid is poured out or filtered, distilled, and the residue titrated either directly with N/10 or N/100 acid (using haematoxylin or methyl-red), or indirectly by first dissolving in excess of acid and titrating back with alkali. In this case iodeosin is mostly used.

(*b*) The alkaloids are first brought into solution as in (*a*), but shaken out with dilute acid, made alkaline, and again transferred to a non-water-miscible solvent, the solvent then being distilled off and the residue titrated as described in (*a*).

(*c*) The alkaloid is extracted with water or dilute acids, precipitated with alkali, and the alkali washed out and then titrated either directly (after previous solution in alcohol) or indirectly. As an example of such a process the estimation of morphine in opium may be cited.

II

GLUCOSIDES

General methods for the isolation of glucosides, with the exception of the groups of saponins, are not known, since their physical and chemical properties vary considerably.

In most cases the process of isolation is begun from an aqueous or alcoholic extract. Since the glucosides are almost always accompanied by enzymes which hydrolyse them [1] the crushed material should be introduced into the boiling solvent (cf. p. 27). In order to avoid hydrolytic action by acids present sufficient calcium carbonate should be added at the

[1] The enzyme can often be excluded by introducing the material into an alcoholic solution of lead acetate.

beginning. The first extract should be filtered hot, since the glucoside sought can often be obtained immediately in a crystalline condition (*e.g.* amygdalin). If there is no precipitate on cooling, be it with the first extract or the subsequent ones, the further extracts sometimes made are filtered cold, since there are glucosides (*e.g.* condurangin) which are precipitated by heat and redissolve in the cold.

As a rule the extracts must be purified before they can be worked out for glucosides. If water has been used for extraction the lead method is usually followed. The tannins and other impurities may also be removed with copper carbonate or copper hydroxide. Sometimes *amalgamated aluminium* [1] (5 gms. in 200 ccs. liquid) or aluminium hydroxide is used for the same purpose.

The purification of the extract can be done according to S. Ghosh (*20*) in the following manner : a powdered mixture of lead chloride (10% of the weight of bark taken) and lead oxide (one third of the lead chloride) is introduced into the aqueous extract and steam is passed through it with vigorous shaking for 5–10 minutes. The solution is neutralised with a 10% solution of sodium carbonate and, after cooling, filtered with the help of a water-pump. Most of the lead is precipitated as carbonate and the remainder is removed with sulphuretted hydrogen.

That the method of shaking may be occasionally used for the isolation follows from the description of the method of Stas-Otto (see p. 22).

In such cases it is often advantageous to saturate the aqueous solution with common salt or some other salt.

The following are methods of procedure which are frequently applicable. The first has served well for the preparation of *cyanogenetic glucosides* and the second for the glucosides of the *digitalis group*.

[1] This is prepared by dipping metallic aluminium for three minutes in a one half per cent. solution of mercuric chloride and then washing.

(1) The material is extracted with alcohol as described on p. 27. From the filtrate the alcohol is distilled off and the residue is taken up with water. The aqueous liquid is treated with lead acetate, the filtrate is freed from excess of lead by H_2S and then evaporated to the thickness of a syrup. The syrup is boiled repeatedly with acetic ether under a reflux condenser, and the glucoside separates out on cooling.

(2) The material is treated in two portions with three times its bulk of cold water and after twelve hours strongly pressed. One of the press cakes is stirred with its own bulk of water, the water is pressed out immediately and the other cake is extracted with the expressed juice. One may also extract a third time in the same way. The combined extracts are purified by the addition of an excess of a concentrated solution of neutral lead acetate, percolation and pressing out of the lead precipitate and precipitating the excess of lead salt with sodium phosphate. The filtrate is shaken out with $CHCl_3$. The $CHCl_3$ extracts are dried with anhydrous Na_2SO_4 and distilled *in vacuo* or simply concentrated and precipitated with petroleum ether.

This method allows of a separation of saponin-like substances (see below) at the same time since these do not dissolve in chloroform (F. Kraft) (*21*).

A special method is necessary for the isolation of *hesperidin* and the glucosides related to it (*22*). The material is treated with a 2% aqueous solution of caustic soda and left with occasional stirring for a few days at room temperature. The lye is decanted off, the material pressed out and again treated with 2% caustic soda. The extraction and pressing are repeated as long as the extract is faintly coloured. The combined alkaline extract is acidified with HCl. The precipitate is washed by decantation and again dissolved in caustic soda. Carbon dioxide is passed through the solution. The precipitate, which is often obtained after some time, is filtered off, washed, dissolved in dilute caustic soda and again precipitated

with CO_2. This process is repeated until the colour of the precipitate shows no essential change and the wash-water is only faintly coloured.

Further purification may be effected by boiling the crude substance with alcohol in order to dissolve out the impurities and then treating with ammonia. Finally, it is again dissolved in dilute caustic soda and precipitated with carbon dioxide.

In a few cases the glucoside may be taken up by an adsorbing material (charcoal, fuller's earth) and again extracted from it as in the following process patented for the isolation of a glucoside from *squills* (*23*). The aqueous extract, clarified if necessary by centrifugalising, is treated with animal charcoal with stirring and shaken for two hours more. The charcoal, which settles down after standing for a short time, is filtered by suction, washed with a little water and dried *in vacuo* at 15°. The glucoside is completely extracted from the charcoal by means of dry hot $CHCl_3$ and the $CHCl_3$ is distilled *in vacuo*. The residue is dissolved in methyl alcohol and the solution shaken with petroleum ether. The methyl alcohol is removed *in vacuo*, the residue dissolved in a little absolute alcohol and the glucoside precipitated by pouring the alcoholic solution into ether. From the adsorption compound with fuller's earth, which can be obtained in an analogous manner, the glucoside can be obtained by extraction with anhydrous hot methyl alcohol and further treatment of the solution as before.

For those glucosides which are precipitated by alkaloid reagents the methods given for the decomposition of the precipitates in the isolation of alkaloids (see p. 46) may be used. Attention may be drawn to the fact that in a few cases an excess of the precipitating agent may tend to redissolve the precipitate formed.

The glucosides with tannin-like properties are prepared according to the methods given for tannins (see p. 111).

Many *glucosides* are present in plants *in combination with*

tannins. These compounds may be obtained according to Wiechowski (*24*) by salting out the aqueous extract and extracting the precipitate with alcohol in order to separate the glucoside compound from the salt. From an aqueous solution of the compound the tannin component can be precipitated with lead acetate and the glucoside then salted out.

Plants often contain several glucosides, and besides these there may be esters of acids with polyhydric alcohols and sugars which behave similarly. The separation in such cases is often attended with great difficulty.

As an example of such a separation (according to K. Freudenberg) (*25*) we may refer to the method by which Gilson (*25a*) isolated *glucogallin* and *tetrarin* from *rhubarb*. Rhubarb is exhausted with cold acetone and the solution evaporated until it has a density of 1·000. Half its volume of a mixture of equal parts of acetone and ether is added to it in small portions with vigorous shaking, and then ether is added (about 1 volume) until the voluminous precipitate first formed settles as a sticky mass. It consists chiefly of the glucosides of oxy-methyl-anthraquinones. The decanted solution is considerably concentrated and the specific gravity again brought down to 1·000 by the addition of acetone. One volume of a mixture of one part of acetone and two parts of benzene is carefully added and then one volume of pure benzene. The oily precipitate contains glucogallin which is crystallised by solution in acetone and precipitation with benzene.

The decanted mixture of acetone and benzene is evaporated until the acetone and most of the benzene are removed. A precipitate is formed thereby which is separated after cooling from the benzene solution containing the methyl-anthraquinone derivative. Hot water extracts a crystalline catechin from the precipitate. The portion of the precipitate insoluble in water contains the *tetrarin*. It is dissolved in acetone, the solution treated with ether and the precipitate removed. The ether-acetone solution is distilled off, the residue dissolved in acetone,

and the solution carefully treated with benzene until a portion is precipitated again. Further fractions are obtained by distilling off the acetone from the mixture in a series of steps. The precipitates are stirred with acetic ether, the crystalline portions of tetrarin collected, and the oily portions submitted to renewed fractionation.

In cases where it is difficult to separate the glucoside from carbohydrates it is advantageous to ferment the latter, where possible, as in the case of cane sugar, glucose and fructose, with yeast.

In order to *free glucosides from the last traces of reducing sugars* the latter may be transformed into the corresponding oxynitriles by addition of hydrocyanic acid and then into the corresponding acids, and the latter precipitated with lead acetate. The aqueous solution is treated with HCN and ammonia and kept until the rotation observed in a polarimeter becomes constant. It is treated drop by drop with a solution of lead acetate until the precipitate does not increase any more, filtered, and the filtrate freed from lead by H_2S (*26*).

On account of their many common properties a special description is necessary of the methods of preparation of the members of the large group of the glucosides known as the *saponins*. If the preliminary test (*d*) (see p. 21) has given the characteristic saponin froth, in order to confirm the presence of a saponin, a small portion of the substance (10–15 gms.) is taken and extracted by boiling with alcohol, filtered hot, and the cooled liquid mixed with ether, without filtering off any precipitate that may be formed on cooling. The precipitate which may be rendered impure by the presence of tannins, salts and sugars, if absolute alcohol and absolute ether have not been used, sticks firmly to the bottom of the vessel in which the precipitation is carried out. In this case the ethereal liquid should be decanted off and the vessel rinsed out a few times with ether. If the precipitate is flocculent it should be placed upon a filter and washed with ether. It is

then dissolved in hot water, adding soda if necessary until it is neutral. If the solution contains saponin it shows the following *properties*: on shaking it gives (after cooling) the characteristic froth of saponin (see p. 21), it emulsifies turpentine oil, fixes mercury, and usually disintegrates red blood corpuscles. On boiling with HCl it produces an insoluble substance (*sapogenin*) and a reducing sugar, and possibly glucuronic or galacturonic acid. After complete hydrolysis the liquid no longer froths.

On evaporating the solution a residue remains which has a harsh taste and when powdered provokes sneezing. Most residues assume a reddish violet colour on treatment with concentrated H_2SO_4.[1] Further, the behaviour of the solution with baryta water on the one hand and lead acetate and basic lead acetate on the other should be investigated.

Since even the physical properties of different saponins may be widely different the directions by which every saponin can be prepared cannot be given. Above all, care has to be taken that a saponin is not altered during its preparation, as in the case of some saponins (*e.g.* Cyclamin, according to Dafert) there is a change merely on prolonged boiling in water. If the saponin possesses *haemolytic action*, as is the case with most of them, the change can be ascertained by a quantitative estimation of the haemolytic action. " If the saponin obtained from a drug has not changed during the preparation then a definite amount of the saponin should possess the same haemolytic action as the amount of drug from which the saponin has been obtained quantitatively " (L. Köhler and O. Dafert) (*27*). The standard is served by the *Haemolytic Index*, that is, the dilution (in " normal saline " solution) at which complete haemolysis is observed.

The estimation of haemolytic index can be done according to the method of L. Kofler and Ph. A. Adam (*28*).

[1] The alcoholic solution of (all ?) saponins gives a precipitate with an alcoholic solution of cholesterin or phytosterin.

As a rule the preparation of pure saponin is facilitated if the material is freed from non-saponin matter by extraction with ether, petroleum ether, etc.

For the preparation of saponins several examples may be given.

1. **Preparation of a saponin soluble in water and in 60% alcohol but insoluble in strong alcohol** (*27*).

10 gms. of the drug powder are heated to boiling several times in a cotton bag, each time with about 300 cc. of distilled water, until exhausted ; the combined extracts are concentrated on the water-bath to about 50 ccs., filtered and concentrated further to the consistency of a syrup. While still hot this is slowly mixed during stirring with about three times its bulk (about 25 ccs.) of hot 60% alcohol and filtered hot by suction from the slight flocculent precipitate obtained. The filtrate is poured drop by drop into 100 ccs. of 96% alcohol. The flocculent precipitate resulting is filtered and dried *in vacuo*. A further quantity of saponin can be precipitated from the filtrate by means of ether.

2. **Preparation of a saponin soluble in water and in strong alcohol** (*29*).

The material is fully extracted with alcohol, the solution evaporated off, taken up with water, and the aqueous solution treated with fuller's earth. This is filtered off and extracted with alcohol. The filtrate is concentrated and the residue again taken up with alcohol. The solution is decolourised by charcoal and treated with an alcoholic solution of oxalic acid. The saponin is obtained from the filtrate.

3. **Preparation of a saponin soluble in water and in methyl alcohol** (Boorsma).

The dried material is boiled or extracted in an extraction apparatus with methyl alcohol. The methyl alcohol is distilled off to a small bulk, precipitated with ether and the precipitate washed with ether. It is then taken up with chloroform-water,

filtered if necessary, and dialysed at least until the dialysate no longer reduces an alkaline copper solution. The solution is evaporated to dryness, the residue taken up with methyl alcohol and again precipitated with ether.

If the saponin is not extracted wholly with methyl alcohol the material can be further extracted with 50% ethyl alcohol. The concentrated liquid is first treated with methyl alcohol and then precipitated with ether (v. d. Haar). The saponin extracted with 50% alcohol may be different from that extracted with methyl alcohol, or one may be the calcium or magnesium compound of the other.

4. **Preparation of a saponin soluble in 70% alcohol but insoluble in water** (*30*).

The coarsely powdered drug is heated under a reflux for two hours with five times its bulk of 70% alcohol. The residue is filtered, pressed and again extracted in the same manner. From the combined filtrates the alcohol is distilled off until about one-third of the original volume is left. The liquid is then poured into an evaporating basin and treated with water until a precipitate is formed. The liquid is evaporated on a water-bath until the alcohol is removed. The precipitate is filtered, the filtrate concentrated further, and any further precipitate obtained combined with the first one. The moist precipitate is dissolved under a reflux in 70% alcohol, the hot solution treated with animal charcoal and digested for half an hour on the water-bath. From the clear liquid obtained by filtration the saponin is precipitated as before with water and after drying (*in vacuo*) crystallised from 96% alcohol.

Separation of Different Saponins from one another

General methods for the separation of saponins from each other, like methods for their isolation, are naturally few. Differences in solubility are of primary importance as a basis.

In special cases the following methods may be used :

1. According to Kobert (*Beiträge zur Kenntnis der Saponinsubstanzen*, 1904, p. 20), an *acid-saponin* can be precipitated out of a solution containing an acid and a *neutral saponin* by salting out with ammonium sulphate. The former is precipitated by first saturating with ammonium sulphate and then boiling for a few minutes. A few saponins, however, which are usually designated as neutral, like cyclamin and chamälirin, are precipitated by this means.

2. For many plants two saponins can be isolated by the lead method (see p. 31), provided one saponin (saponinic acid) is precipitated by lead acetate and the other by basic lead acetate. Since the saponins are strongly adsorbed by lead sulphide the lead precipitates [1] are usually decomposed by dilute sulphuric acid and the little lead that goes into the filtrate is removed by H_2S. The liquid filtered from PbS is evaporated to the consistency of an extract and the residue boiled with alcohol, or, when it is highly coloured, with a mixture of one part of absolute alcohol and four parts of chloroform. From this solution the saponin is precipitated by ether and dried over sulphuric acid.

Since a saponin may be changed even by the action of basic lead acetate the second saponin from the filtrate from neutral lead acetate should be isolated by some other method. In case the passing of H_2S cannot be avoided it should be noted that the acetic acid set free may likewise change the saponin.

Purification of Saponins

The saponin prepared according to one of the methods described above will be pure only in exceptional cases ; as a rule it will contain impurities, almost always inorganic matter, and also carbohydrates, tannins, colouring matters, etc. A

[1] As the lead precipitates give up the saponin easily to water, or since they are partially soluble in it, one should wash them only once or twice with water, then with dilute alcohol, and finally with 95% alcohol.

purification is sometimes attempted by repeating the treatment used in the method of preparation ; but it is seldom that a pure saponin can be obtained in this way.

The saponin is freed from inorganic matter and other crystalloids by dialysis,[1] or, still better, by *electro-dialysis*, using the apparatus of W. Pauli, in which a platinum net-electrode is used as anode and a similar silver electrode is used as cathode (*27*).

To free the saponin from other colloids one of the following methods can be used in addition to the lead method (see above). It should, however, be noted that the saponin may be altered thereby, as seen by the diminution of the haemolytic index, etc. The fundamental chemical changes involved in this alteration have not yet been clearly worked out. If, in spite of these changes, such a process of purification is used, it is done because in many cases it may be more advantageous for further chemical investigation to possess a pure saponin, even if altered, than an impure unchanged one. In all cases the purified saponin may be subjected to dialysis or electrodyalysis.

1. **The magnesia method** depends upon the fact that tannins, saponins, colouring matters, etc., form compounds with magnesia, but only in the case of saponins is the compound with magnesia decomposed by boiling alcohol, the saponin then being taken up by the alcohol. The aqueous solution of the saponin is evaporated to dryness on the water-bath after being mixed with ignited magnesia or with freshly precipitated magnesium hydroxide (Flieringa). The mass is powdered as finely as possible and boiled with alcohol under a reflux condenser. By fractional precipitation with ether that portion of saponin is precipitated from the alcoholic solution which remained dissolved in the cooled liquid. One should precipitate fractionally in order to obtain a preparation as free as possible from ash, since the first precipitate usually

[1] On the behaviour of saponins on dialysis see L. Kofler and A. Wolkenberg, *Biochem. Zeitschr.*, 160, 398, 1925, and Pfau, E., *Apoth. Ztg.*, 40, 100, 1925.

contains more inorganic constituents than the subsequent ones.

2. **The baryta method.** The concentrated aqueous solution of the saponin is treated with a saturated solution of baryta.[1] The precipitated baryta saponin is washed with baryta water, in which it is insoluble, suspended in water and decomposed by CO_2 into barium carbonate and saponin. The saponin solution is evaporated, the saponin is taken up with alcohol and obtained either by evaporation, or, better, by fractional precipitation with ether (see above).

3. **The lead hydroxide method.** The saponin in alcoholic solution is boiled for some hours with lead hydroxide (freshly precipitated and washed with alcohol) under a reflux condenser. The lead remaining in the filtrate is precipitated with carbon dioxide, the last traces removed by H_2S and the filtrate treated with ether as in 2.

4. **The copper method.** The aqueous extract is treated with copper carbonate, the tannins (see p. 113) and other impurities are precipitated, and the filtrate, made alkaline with NaOH, is either boiled with copper hydroxide or a solution of copper sulphate is added to the hot liquid kept alkaline in reaction. The saponin is completely precipitated by this method. The copper compound formed is washed and decomposed either by H_2S or by an amount of HCl or H_2SO_4 insufficient for complete decomposition, and the saponin separated from the copper salts by dialysis.

5. Of doubtful value is the process of Merck (*31*) in which the saponin is precipitated by tannin and the precipitate decomposed by lead hydroxide. Apart from the fact that only a portion of the saponin is precipitated by tannin there are carbohydrates and other glucosides which behave quite similarly.

The quantitative estimation of a saponin can be performed by using a suitably small quantity of the drug and carrying out the method of preparation in a quantitative manner. If the

[1] In many cases it is advisable to add $CaCl_2$ before the addition of baryta water in order to precipitate the non-saponins which give insoluble compounds with alkaline earths.

haemolytic index is known, or has been determined, a quantitative estimation can be based upon this.

For those saponins which give a precipitate with baryta water this property can be utilised.

The drug is boiled three times with water, the extracts are concentrated to a small volume, treated with alcohol and filtered. The precipitate is boiled repeatedly with dilute alcohol ; the alcoholic decoction is filtered hot and combined with the first filtrate. The alcohol is distilled off, the residue taken up with water, evaporated to a small bulk, treated with a saturated solution of baryta, and the saponin baryta is collected on a tared, dried filter. The precipitate is washed with a saturated solution of baryta until the latter runs out colourless. The precipitate is then dried first in a drying oven, and, finally, in an air oven at 110° to constant weight. The weight, after deducting the weight of the filter, gives the amount of saponin baryta. The saponin baryta is ignited with the filter, and from the mass of the residue ($BaCO_3$) the corresponding barium oxide calculated and this weight deducted from the amount of baryta saponin. Another process depends upon weighing the water-insoluble sapogenin formed by hydrolysis with acids (see below). The method can naturally be used only when the amount of sapogenin obtained by hydrolysing the pure saponin is known.

The quantitative estimation of glucosides, not belonging to the class of saponins, can generally be performed in the same way as with saponins. Thus, either the method of preparation may be carried out quantitatively, or, where possible, the glucoside may be precipitated as an insoluble compound. If the hydrolysis takes place quantitatively, and has been sufficiently investigated, a product of hydrolysis may be estimated. For example, the hydrolysis with emulsin often proceeds almost quantitatively so that it suffices to estimate the sugar formed under its influence. In many cases the estimation of the volatile products of hydrolysis can be used for the

estimation of the glucoside, as in the case of cyanogenetic and mustard oil glucosides.

The hydrolysis of the glucosides may be carried out with water, acids or enzymes. If the hydrolysis is to be performed with water alone a temperature above 100° C. is usually necessary. The hydrolysis should then be carried out in a pressure-bottle or a sealed tube. For glucosides difficult to hydrolyse it is better to add HCl or H_2SO_4. Many glucosides are hydrolysed by boiling (often by simply warming on the water-bath) with 1 to 10% acid. Hydrochloric acid often hydrolyses better than sulphuric acid. In a few cases fine products of hydrolysis are obtained by passing dry HCl gas through a hot alcoholic solution of the glucoside. It should, however, be remembered that there is a possibility in this case that the product of hydrolysis may be ethylated (esterified).

To obtain the product of hydrolysis from the aqueous liquid it is separated out by shaking with ether, etc. ; if the substance is insoluble in water and ether it may be obtained by filtration ; the appearance of a volatile product of hydrolysis may be detected by the smell and by distillation. In order to isolate the products of hydrolysis which cannot be isolated thus, especially the sugars, the acids are removed, excess of H_2SO_4 by $BaCO_3$ and of HCl by moist $AgCO_3$ or silver acetate. For the isolation and identification of sugars see pp. 133 *et seq.* It may, however, be remarked here that among the products of hydrolysis one may often find mixtures of sugars, like glucose and galactose or glucose and rhamnose, and that glucose, from which the name glucoside has been derived, may be altogether absent from the products of hydrolysis and may be replaced by other sugars (rhamnose, quinovose).

It should also be noted that sugars may be absent altogether and may be replaced by the corresponding acids (glucuronic acid, galacturonic acid) (see p. 149).

In order to determine what sugar is present one should

first test for hexoses (see pp. 133 *et seq.*) and then for fructose, pentoses (see p. 145) and methyl-pentoses (see pp. 146 *et seq.*).

In the analysis of a glucoside, in which, as is often the case, it is necessary to identify *rhamnose and glucose* occurring together, one may proceed as follows : The osazone is first prepared as described on p. 138 and it is then treated with acetone. The glucosazone (see p. 138) remains behind undissolved and is recrystallised from 70% alcohol. The rhamnosazone (M.P. 185° C.) is obtained by distilling off the acetone and recrystallising from benzene.

For the identification of other sugars in the presence of one another see pp. 133 *et seq.*

If a glucoside-splitting enzyme is present in a plant containing a glucoside, like myrosin in black mustard, the hydrolysis should be attempted with the enzyme. Alternatively an aqueous extract of yeast is frequently used, or emulsin or rhamnodiastase, but none of these is applicable universally. In this connection it may be mentioned that amygdalin is hydrolysed by emulsin to sugar, benzaldehyde and HCN ; while yeast extract hydrolyses it to mandelonitrile glucoside and glucose. Moreover, it has been established by the researches of E. Fischer that emulsin, which hydrolyses β-methyl glucoside, leaves the α-compound intact, while an enzyme from yeast behaves in the opposite manner.

III

BITTER SUBSTANCES

There is no general method for the preparation of bitter substances, a non-homogeneous group of substances with a bitter taste, which are neither glucosides nor alkaloids. In many cases one may proceed as in the preparation of glucosides.

As examples *the preparation of picrotoxin and quassiin* (according to E. Schmidt) may be given.

For the preparation of *picrotoxin* the coarsely powdered Cocculus indicus, freed from the main bulk of fat by hot-pressing, is boiled repeatedly with water, the percolated hot extracts treated with a solution of lead acetate sufficient for precipitation, the filtrate freed from lead by H_2S and the filtered liquid concentrated to a small volume. The crystalline mass, which separates out on standing for several days, is freed as far as possible from the mother liquor by filtering by suction and washing with a little cold water. It is then purified by recrystallisation first from boiling water and then from boiling strong alcohol with the use of a little animal charcoal.

To prepare *quassiin*, the aqueous extract of the wood is evaporated to two-thirds of the weight of the quassia wood taken and precipitated with a solution of tannin. The washed precipitate is stirred up with lead carbonate and the mixture is dried on the water-bath. The residue is boiled repeatedly with alcohol, the alcoholic liquid concentrated, and the quassiin which is precipitated is crystallised from dilute alcohol or from a mixture of alcohol and ether.

IV

COLOURING MATTERS

The majority of the colouring matters occur as glucosides. To obtain these glucosides, from which the true colouring matters are formed on hydrolysis, one may proceed in most cases as described in the chapter on glucosides.

As an example *the preparation of quercitrin from quercitron bark* may be described: The powdered bark is boiled with five to six parts of 85% alcohol for six hours. The filtered extract is concentrated to half the bulk and then mixed with glacial acetic acid and an alcoholic solution of lead acetate (avoiding excess as far as possible). The filtrate is freed from lead by H_2S, and the liquid, filtered from PbS, evaporated to dryness. The residue is taken up with alcohol. From the

filtrate the quercitrin is precipitated with water and purified by crystallisation from alcohol.

Although they do not contain any nitrogen, colouring matters like anthocyans have the peculiarity of possessing basic properties and may be isolated as salts.

One may proceed in such cases according to the method described by Willstätter and Mallison (*32*) for *the preparation of colouring matters from mountain-cranberry*. The skins of the berry are extracted with glacial acetic acid and the colouring matter precipitated from the solution with ether. The precipitate is dissolved again in glacial acetic acid and precipitated with ether in two fractions. The precipitate of the second fraction is washed with ether and dissolved in water. The colouring matter is precipitated from the solution as picrate by an aqueous solution of picric acid, the first precipitate being filtered off and the filtrate allowed to crystallise in an open dish. Since the crystals are admixed with potassium picrate they are treated with methyl-alcohol in which only the salt of the colouring matter is easily soluble. By precipitating with ether and recrystallising from water the picrate can be obtained pure. The methyl alcoholic solution of the picrate is treated with methyl-alcoholic hydrochloric acid and precipitated with ether. The precipitate, *idaein chloride*, is freed from picric acid by washing with ether. For purification the chloride is dissolved in water, treated with concentrated HCl, filtered from the flocculent precipitate and allowed to crystallise after the addition of alcohol.

In some cases it has been possible to obtain the colouring matters in crystalline condition by treating the extracts with alkalies, either an aqueous solution being treated with aqueous KOH (saffron) or by a solution in petroleum ether being treated with methyl alcoholic potash (capsicum).

Compare P. Karrer, *Helv. Chem. acta* 10, 397, 1927 (saffron), and L. Zechmeister, and L. von Cholnoky, *Annal. Chem.* 454, 1, 1927 (capsicum).

Separation of Anthocyanidines and Tannins according to Jonesco (*32a*)

1. After a preliminary treatment of the dry, finely powdered material with benzine, benzene or petroleum ether, the tannin is extracted with ether, moistening the powder from time to time with fairly strong HCl. The anthocyanidines remain in the residue.

2. The material is macerated for a week with concentrated HCl, which dissolves the anthocyanidine.

(*a*) *Isolation of anthocyanidine.* The filtrate is left for a few days in a large porcelain dish under a bell-jar. The precipitate of the colouring matter which settles down is dissolved in a little alcohol and treated with amyl alcohol, which keeps the anthocyanidine in solution during its further treatment. The amyl alcoholic solution is washed several times with acidified water, then with a solution of sodium acetate, which takes up the anthocyanine and traces of colouring matter, and finally washed 2–3 times with HCl. The solution is then filtered and allowed to evaporate. The residue is dissolved in methyl alcohol and the anthocyanidine precipitated with one-third of its volume of HCl. The liquid above the precipitated colouring matter is decanted off, the colouring matter washed rapidly with HCl and dried.

(*b*) *Isolation of tannins.* The residue remaining after filtering off the liquid containing HCl is extracted with ether, which takes up the tannins.

For further information see *Compt. rend. soc. biol.* 95, 129, 1926.

V

FATS AND FATTY OILS

The fats and fatty oils are obtained either by pressing in the cold or hot (for a better yield) or by extraction with fat solvents

(petroleum ether, trichloro-ethylene, ether, carbon disulphide, etc.) and subsequent distillation of the solvents.[1] These methods usually yield slightly different products.

The physical and chemical properties of the product will show whether fats or fatty oils are present. It should exhibit a " fatty " nature, float upon water, and produce a " grease-spot " on paper ; it will not dissolve in water, or dilute alcohol and seldom in strong alcohol. It should further, as a rule, be saponifiable, *i.e.* when treated with alkali (see below) it should produce glycerine and soaps, *i.e.* alkali salts of higher fatty acids.[2]

To ascertain whether an oil is saponifiable *the micro-chemical method* is the most rapid. A small drop (at most 2 mg.) of the oil is placed upon a slide by means of a needle. A drop of a saturated alcoholic solution of KOH or NaOH [3] is placed upon it with a drawn-out glass rod, and it is at once covered with a cover glass in order to avoid the formation of carbonate as far as possible. If it is a solid or semi-solid fat a corresponding amount is placed upon the slide and melted over a small flame and worked further as before. If the fat is saponifiable, soap crystals appear, usually in a short time, and can easily be recognised by means of a microscope (*33*).

[1] It is important, not so much for the systematic investigation but for the determination of the constants, to remove the solvents completely. This is best done by heating at 100° *in vacuo* for some time.

If substances which do not belong to the class of fats go over to the fats they will vitiate the determination of the physical properties and of the usual constants. It will, therefore, be necessary to remove them ; alkaloids, for example, by shaking the oil (or its solution) with dilute HCl. The residual acid is then removed by shaking with water and the residual water by means of anhydrous sodium sulphate. For the removal of the solvents see above.

For the rare case in which a hydrocyanic acid compound goes over to the oil see L. Rosenthaler, *Schweiz. Apothekerztg.*, 58, 17, 1920.

[2] The case in which a vegetable fat contains no glyceride will only seldom occur ; one should, however, consider this possibility specially in underground organs.

[3] To prepare the lye the hydroxide sticks are washed with a little water, then with absolute alcohol after removing the water as far as possible. So much absolute alcohol is then added that a portion of the hydroxide remains undissolved. The liquid is then decanted off or filtered.

The investigation usually starts with the determination of the *physical properties*: odour and taste, melting point, solidifying point, colour, consistency, density, solubility, refractive-index, polarisation and viscosity.

In addition there are a few empirical tests with reagents which give *colour reactions* (*34*).

1. *Welman's Reaction.* One gm. of the clear fat, melted and filtered if necessary, is dissolved in 5 ccs. of chloroform in a thick-walled graduated test tube provided with a glass stopper, treated with 2 ccs. of a freshly prepared solution of phosphomolybdic acid or its sodium salt, a few drops of HNO_3 added and shaken vigorously.

2. *Bellier's Reaction.* Five gms. of fat, melted and filtered if necessary, are shaken vigorously for 5 seconds with 5 ccs. of colourless nitric acid (sp. gr. 1·4) and 5 ccs. of a cold-saturated solution of resorcin in benzene in a thick-walled test-tube provided with a glass stopper.

Only colourations observed during the shaking or 5 seconds afterwards should be taken into consideration.

3. *Serger's Reaction.* Five ccs. of oil or liquefied filtered fat are placed in a thick-walled graduated test-tube provided with a glass stopper and dissolved in 10 ccs. of ether. One c.c. of freshly prepared Serger's Reagent [1] is placed below the layer (holding the test-tube in an inclined position) and then shaken vigorously for a very short time. The lower layer is examined 15 minutes after the separation of the layers.

4. *Kreis's Reaction.* Equal volumes of fat and HCl (sp. gr. 1·19) are first shaken together and then with one volume of a 1% solution of phloroglucin in aldehyde-free ether.

5. *Wiedmann's Reaction.* Equal volumes of fat and a 1% solution of fat in acetone are shaken together and then with 2–3 gms. of sulphuric acid.

6. *Baudouin's Reaction.* Five ccs. of fat or oil, melted and filtered if necessary, are shaken vigorously for at least half a minute with 10 ccs. of HCl (sp. gr. 1·19) and 0·1 c.c of a 1% solution of furfural in alcohol in a strong test-tube provided with glass stopper.

[1] Ten ccs. of conc. H_2SO_4 and 0·1 gm. of very finely powdered sodium molybdate are taken in an upright cylinder, provided with a glass stopper, and shaken vigorously for two minutes. The reagent can be used after five minutes; after half an hour it is no longer reliable.

7. *Halphen's Reaction.* Five ccs. of oil are heated with the same volume of amyl alcohol and 5 ccs. of 1% solution of S in CS_2 in a test-tube under a reflux condenser on the water-bath for 15 minutes. If there is no colouration 5 ccs. more of the sulphur solution are added and heated for 15 minutes more.

8. *Becchi's Reaction.* Reagents : (I) A solution of 1 gm. $AgNO_3$ in 200 gms. alcohol is treated with 0·1 gm. HNO_3 and 40 gms. ether and filtered. (II) A mixture of 100 gms. amyl alcohol and 15 gms. of rape oil. If 1 c.c. of (I) is heated with 100 ccs. of (II) for 15 minutes on the water bath there should be no brown or black colouration.

To carry out the reaction 5 ccs. of the filtered fat or oil are taken in a flask with 10 ccs. of absolute alcohol and boiled under a reflux with 10 ccs. of (II) and 1 c.c of (I) for 15 minutes, the flask being protected against direct daylight.

9. *Hauchecorn's Reaction.* Equal volumes of oil and HNO_3 (sp. gr. 1·375) are well shaken and observed after 24 hours.

10. *Soltsien's Reaction.* Mix 2 to 3 parts of fat, melted if necessary, with 1 part of a solution of stannous chloride in HCl, shake vigorously so that an emulsion is formed, and place the vessel in a hot-water bath up to the layer of stannous chloride.

11. *Crace-Calvert's Reaction.* Ten ccs. of oil or melted fat are shaken with 2 ccs. of a mixture of equal parts of conc. H_2SO_4 and conc. HNO_3.

12. *Cavalli's Reaction.* Bring together, without mixing, equal parts of oil and a mixture of 3 parts of HCl with 1 part of HNO_3.

The *Elaidin Reaction* should also be carried out.

Usually this reaction is carried out by placing the oil with an equal volume of HNO_3 together with a few pieces of copper wire in a test tube.

Alternatively 2 gms. of the oil and 10 ccs. of nitric acid are brought together, 1 gm. of sodium nitrite is added in small portions and allowed to stand in a cool place. When the test is positive the oil sets to a solid mass, since the liquid glyceride of oleic acid is converted into the solid glyceride of elaidic acid.

The systematic determination of chemical constants, which is

usual in the analysis of fats, may also furnish information concerning the qualitative composition, quite apart from its significance in quantitative work. The *acid value* shows the presence of free fatty acids. An *iodine value* is given only by unsaturated compounds. If there is an *acetyl value* [1] for free acids it can only be due to hydroxy-acids.

In working with acids one may use the following method of acetylation suggested by the German Commission to secure a uniform method of investigation for the fat industry :

Six to eight gms. of the total fatty acids are boiled with double the quantity of acetic anhydride for 2 hours in an acetylating flask (condenser with ground glass joints). The mixture is boiled for half an hour with 500 ccs. of water, transferred to a separating funnel and washed several times with very hot water [2] until neutral to litmus. The separated acetyl product is filtered through a dry filter.

To estimate the acetyl number, according to Croner (*35b*), about 2 gms. of the acetylated product (weighed accurately) are dissolved in about 5 ccs. of neutral alcohol and titrated in the cold with N/2 alcoholic caustic potash (this gives the *acetyl-acid-number*, or number of milligrams of KOH required to saturate the free acetylated acids). It is then saponified with about 25 ccs. of approximately N/2 alcoholic potash for half an hour on a boiling water-bath, the alcohol removed, the residue transferred quantitatively with the help of water to a long-necked flask (Kjeldahl flask) and the fatty acids set free with excess of dilute sulphuric acid.

After setting up a Kjeldahl flask the acetic acid set free is distilled in steam into a 1-litre Erlenmeyer flask previously washed with

[1] To avoid the mistake which arises from the fact that fatty acids may form acid-anhydrides with acetic anhydride it is better to use, according to A. Grün (*35*), the methyl- or the ethyl-ester, which is prepared by the well-known method (see also p. 74, note 1) of passing HCl gas into a solution of the acid in absolute alcohol. The excess of alcohol is evaporated off, the residue taken up with ether, the ether solution washed repeatedly with water, then with a solution of sodium carbonate, dried with anhydrous sodium sulphate and the ether driven off.

The ester can also be obtained directly from the fat by heating it with methyl- or ethyl-alcohol and 1 to 2% H_2SO_4 or HCl.

[2] According to Holde and Bleyberg (*35a*), the acetic anhydride is removed by washing the reaction product dissolved in benzine (at 70°–80° C.) first with about 50% acetic acid and then with hot water.

neutral alcohol, and the distillate (600–700 ccs.) titrated with N/10 KOH (using phenolphthalein).

The acetyl value (A.V.) $= \frac{5{\cdot}611 \times b}{a}$, where a is the acetylated product and b is the number of ccs. of N/10 KOH required.

If an acetyl value is found with oils which contain no hydroxy-acids it may be due to mono- or di-glycerides or to higher alcohols.

In order to test the *drying-power* of an oil (and from this to detect the presence of higher unsaturated fatty acids) it is spread in a thin layer over a glass plate. A drying oil will thereupon gradually dry up to an elastic skin, the process requiring up to two or three weeks.

Whether an oil is a drying one or a semi-drying one may be determined by *the hexabromide-test of Hehner and Mitchell.* One to two gms. of oil are dissolved in 40 ccs. of ether to which a few ccs. of glacial acetic acid have been added. The solution, cooled in ice, is treated with bromine and allowed if possible to stand over night in ice. If there is a precipitate it is filtered by suction, best with the suction arrangement of Hehner and Mitchell (*36*), washed once with 10 ccs. of ether at 0° and dried to constant weight. The formation of a precipitate (probably a mixed glyceride of which the acidic portion consists partly of stearic acid hexabromide) shows the presence of linolenic acid and therefore of a drying oil.

The object of the complete investigation of a fat, besides determining the nature of the secondary constituents (sterols, higher aliphatic alcohols and hydrocarbons) and of the glycerine, is either to determine all the acids present in the glycerides or to isolate the glycerides and investigate them further.

For the *separation of glycerides* the process of Holde and Stange (*37*) and Bömer (*38*) may be referred to.

For the separation of glycerides by distillation in high *vacuo* see A. Bömer, and J. Baumann, *Zeitschr. f. Untersuch. d. Nahrungs- u. Genussmittel*, **40**, 97, 1920.

If the isolation of glycerides is omitted one may begin the further investigation with the saponification of the fat.

The saponification [1] *and separation of sterols* can be accomplished according to Bömer (*39*) in the following way :

100 gms. of fat are melted on the water-bath in an Erlenmeyer flask of 1–1½ litres capacity and saponified with 200 gms. of alcoholic KOH (200 gms. KOH in one litre of 70% alcohol) under a reflux condenser on a boiling-water bath. The flask is frequently shaken vigorously until the contents become clear, and warmed again for ½ to 1 hour on the water bath with occasional shaking.

The warm soap solution is taken in a separating funnel (capacity about 2 litres) containing 300 ccs. of water, the residue in the flask washed with 300 ccs. of water and poured into the funnel. After sufficient cooling it is shaken vigorously for ½–1 minute with 800 ccs. of ether and the ether is removed after the separation of the two layers. The shaking is repeated two or three times with 300–400 ccs. of ether, the ethereal solution is filtered (to remove the soap solution) and the ether distilled off from an Erlenmeyer flask after adding a few pieces of pumice stone. The alcohol still present in the flask is removed by dipping the flask in a boiling-water bath and blowing in a current of air. The residue is again heated for 5–10 minutes with 10 ccs. of the same solution of KOH (see above) under a reflux condenser in order to saponify any fat remaining behind, the flask is again emptied into a separating funnel, washed with 20–30 ccs. of water, and, after cooling, the contents of the separating funnel are shaken twice with 100 ccs. of ether. After separation of the aqueous-alcoholic layer the ethereal solution is washed three times, each time with 10 ccs. of

[1] Instead of saponifying (hydrolysing) one may also "alcoholyse." The fat is treated with an equal volume of methyl alcohol and so much methyl-alcoholic solution of HCl that the HCl content of the mixture amounts to about 1·5%. It is then heated under a reflux condenser with much shaking until there is no more precipitate of the unchanged fat (glyceride) with water. (Test for glycerine, see p. 78.) The methyl esters of fatty acids are then precipitated with water, washed until the methyl alcohol, glycerine and HCl are removed, and the esters are submitted to fractional distillation. The single fractions can then be saponified and the fatty acids obtained as described for direct saponification.

water, filtered (to remove the water) into a small beaker and the ether allowed to evaporate. The residue contains sterol and also other non-saponifiable components.

Instead of shaking out the sterol with ether one may also extract with the same solvent in an extraction apparatus according to Heiduschka and Gloth (*49*).

According to Fritzsche (*41*) *free sterols* (as contrasted with the esters) can be *separated from the fats* in the following way : 50 gms. of melted, clear, filtered fat are treated in a 150 c.c. beaker with 20 ccs. of a 1% solution of *digitonin* in alcohol. The mixture is warmed up to 60°–70° and stirred briskly for 5 minutes with a mechanical stirrer, the liquid being kept at the above temperature during the stirring.

In the case of liquid and semi-solid fats the fat is first filtered by suction through an easily permeable filter paper placed on a Buchner funnel 50 mms. in diameter, and the residue freed from fat by washing six times with ether under gentle suction, each time with 5 ccs. of ether. In the case of solid fats one should add after the stirring 20 ccs. of chloroform to the still warm liquid, filter under suction and wash the residue twice, each time with 5 ccs. of warm chloroform, and then six times, each time with 5 ccs. of ether.

The *digitonide* may be decomposed by heating with xylol, which dissolves out the sterol. Or one may acetylate by heating with acetic anhydride, remove this by heating on the water bath with the help of a current of air and obtain the sterol acetate by recrystallising the residue from alcohol. The digitonin process may also be used in the following way to obtain the sterol (esterified and non-esterified). Saponify and separate the fatty acids from the soaps by an acid. To the fatty acids from 50 gms. of fat add 25 ccs. of a 1% solution of digitonin in alcohol, keeping the temperature at 70° C. If there is a precipitate add 20 ccs. of chloroform to the still hot mixture, filter by suction on a warm filter and remove the fatty acids by washing with warm chloroform and ether.

The closer investigation (*42*) *of the non-saponifiable constituents* may be carried out by heating them for 1–2 hours with an equal weight of acetic anhydride under a reflux condenser. In the absence of hydrocarbons this leads to complete esterification and solution ; on cooling a portion of the ester may separate out ; the separation becomes complete on treatment with water. The esters of the higher fatty alcohols and those of the phytosterols are separated by their behaviour towards alcohol. The esters of fatty alcohols, the presence of which is shown by the wax-like components of the non-saponifiable fats, dissolve easily in boiling alcohol and remain dissolved in it on cooling, while the phytosterol acetate is soluble with difficulty in alcohol and is precipitated on cooling the hot solution. The esters of fatty alcohols may be precipitated by adding water to the alcoholic solution or saponified by boiling with caustic alkali. On diluting the saponified solution with water the fatty alcohols are again precipitated.

The aliphatic alcohols are also distinguished from the phytosterols by heating with soda-lime. The phytosterols remain unchanged thereby, while the fatty alcohols yield acids with the same number of carbon atoms.

For the separation of alcohols and hydrocarbons see also p. 93.

Separation of Solid and Liquid Phytosterols (Matthes) (*43*)

The crude phytosterol is kneaded in a basin with about the same bulk of low-boiling petroleum benzine and placed well covered for a long time over ice. The crystals which separate out are quickly drained on a filter plate and washed with a few drops of cooled petroleum benzine.

The filtrate is concentrated, and, after cooling, kept in ice in the same way as before with petroleum benzine, and the solid portion separated by filtration and washing. This process is repeated until no more crystals are obtained on longer cooling.

The solvent is removed from the petroleum benzine solution and the residue is treated with cold absolute alcohol. It is allowed to stand for several days. If crystals separate out they are filtered by suction and washed with a little cooled alcohol. This treatment with alcohol is repeated until no more crystals separate out, and the residue from the alcoholic solution may be again submitted to the treatment with petroleum benzine.

Separation of Phytosterols according to Windaus and Hauth (*44*)

They are first acetylated according to page 75. One gm. of dry phytosterol acetate is dissolved in 10 ccs. of ether and treated with 2·5 ccs. of a 5% solution of bromine in glacial acetic acid. The precipitate (phytosterol acetate bromide) that may be formed is filtered by suction and washed with cooled ether. If the filtrate is treated with a little alcohol, and then with so much water that a turbidity is produced, the phytosterol acetate bromide remaining in solution is precipitated [1] and should be washed with water containing alcohol.

To regain the phytosterol from the acetate bromide it is first debrominated by heating 1 gm. of the compound with 1 gm. of zinc dust and 20 gms. of glacial acetic acid for a few hours under a reflux condenser. From the hot filtered solution the phytosterol acetate is precipitated by water and recrystallised from alcohol. It is then saponified by heating with 30 ccs. of 5% solution of KOH in alcohol on the water bath under a reflux condenser for several hours. The phytosterol is precipitated from the solution with water and shaken out with ether. The ethereal solution is washed with water and dried with anhydrous Na_2SO_4. The ether is then distilled off and the residue recrystallised from alcohol.

[1] The acetate bromide which is precipitated and that which is separated by water are derived from different phytosterols : the former belongs to the type of stigmasterols, the latter to sitosterols (both from Kalabar seeds).

Concerning *the separation of unsaturated sterols from the saturated* ones see R. J. Anderson, and F. P. Nabenhauer, *Jour. Amer. Chem. Soc.*, 46, 1957, 1924 ; reference in *Zeitschr. f. Untersuchg. der Lebensmittel*, 53, 183, 1927.

The phytosterols are characterised by their melting point and their (acetyl- and benzoyl-) esters. They also give various colour reactions which are similar to those of cholesterols occurring in animal fats.

Colour reactions are obtained as follows :

1. When phytosterol is dissolved in chloroform and concentrated H_2SO_4 added. H_2SO_4 and $CHCl_3$ show colour reactions ; the latter is frequently coloured blood-red (Hesse's reaction).

2. When it is treated with a mixture of 1 part of water and 5 parts of H_2SO_4 (Moleschott's reaction). Red to violet colours may be formed. If some iodine solution is also added other colourations are obtained.

3. When it is dissolved in hot acetic anhydride and a few drops of H_2SO_4 are added to the cooled solution. Blue colourations may be obtained (Liebermann's reaction).

4. If it is treated with a mixture of 9 parts of trichloracetic acid and 1 part of water or heated with liquid trichloracetic acid up to the boiling point. Colouration red to violet (Hirschsohn's reaction).

5. If it is evaporated with concentrated HCl and $FeCl_3$. The residue shows red or blue colouration after washing with water (Mach's reaction).

After removing the non-saponifiable constituents the ether and alcohol are removed by evaporation and the aqueous solution salted out with common salt.[1] The precipitate

[1] Instead of salting out the fatty acids may be obtained by adding dilute sulphuric acid and shaking with ether. The ethereal solution is washed to remove the sulphuric acid, dried with anhydrous sodium sulphate and freed from ether by distillation in a current of hydrogen. The fatty acids may also be obtained without shaking with ether. They are either precipitated in the cold and filtered or they are precipitated hot, cooled, and the aqueous liquid is separated from the layer of fat.

consists of soaps, *i.e.* the sodium salts of fatty acids ; the liquid contains glycerine and possibly the sodium salts of lower fatty acids.

To test the aqueous liquid for fatty acids that may be present in it it is acidified with H_2SO_4 and distilled fractionally in a current of steam. For identification the fractions are saturated with $AgCO_3$, and the silver salts formed are tested by elementary analysis and estimation of the silver.

If the liquid remaining after distillation is neutralised (or if a portion of the liquid not used for distillation is used), and evaporated as far as possible on the water bath, the *glycerine* can be extracted from the residue by a mixture of 3 parts of 95% alcohol and 1 part of ether, and it remains behind as a syrup after evaporating off the ether and alcohol. The syrup should taste sweet, mix with water and alcohol and show the following reactions :

1. Heated with copper sulphate and NaOH solution the liquid (or when copper oxide is formed, the filtrate) should be blue.

2. On heating with potassium bisulphate the vapour of acrolein with a penetrating smell is formed. It should be passed through a doubly-bent glass tube into water. The aqueous solution should reduce ammoniacal silver nitrate solution, colour a solution of fuchsine sulphur dioxide red and also show the following reactions : (*a*) On the addition of a little sodium nitroprusside and then a secondary base (dimethyl amine or piperazine) there is a blue or (with less acrolein) a green colour. (*b*) With a solution of orcin in strong HCl flakes are obtained.

The soap is dissolved in water and decomposed with dilute sulphuric acid. *The higher fatty acids* are insoluble in water and are precipitated while the lower fatty acids remain in solution when sufficiently dilute and may be separated from one another by fractional distillation. From the mixture of the precipitated fatty acids, *acids up to capric acid can be easily*

distilled in steam, while the next higher members distil with more difficulty. The separation of the non-volatile fatty acids depends partly upon the different behaviour of their salts towards solvents and partly upon the principle of fractional precipitation, crystallisation and vacuum-distillation of the acids or their esters.

With the fatty acids one should first determine the molecular weight and the iodine value, and then, if part of the fatty acid present is unsaturated, proceed with the separation of the saturated from the unsaturated fatty acids.

Separation of Liquid Unsaturated Acids from the Unsaturated and Saturated Solid Fatty Acids

1. **Bremer's process** (*45*). An aqueous solution of zinc acetate is allowed to flow into a hot solution of the fatty acids neutralised with alkali in the presence of phenolphthalein. The precipitate is filtered off, washed and dried as far as possible by pressing between filter papers. It is then gently heated with anhydrous ether for $\frac{1}{4}$ to $\frac{1}{2}$ hour under a reflux condenser. The ether dissolves the zinc salts of the unsaturated acids. To obtain the acids [1] the ethereal solution is shaken with dilute HCl, the HCl run off, the ethereal solution washed repeatedly with boiled water until free from HCl, and dried with anhydrous Na_2SO_4. The ether is then distilled off in a current of hydrogen.

The zinc salts insoluble in ether are washed repeatedly with ether, warmed with HCl, cooled and shaken out with ether, and treated further as in the case of unsaturated fatty acids, omitting only the passing of a current of hydrogen. The saturated fatty acids are obtained in this way (still contaminated with unsaturated).

[1] Mathes and Roth (*48*) distil off the ether in a current of hydrogen under reduced pressure, dissolve the zinc salts in benzene and decompose them in a separating funnel with warm dilute H_2SO_4. In other respects they proceed as above.

2. **Method of Tortelli and Ruggeri** (*46*). The soap solution, obtained by saponifying 20 gms. of oil with 50 ccs. of alcoholic KOH (4 gms. KOH in 50 ccs. of 90% alcohol), is exactly neutralised with 10% acetic acid, after adding 3 drops of phenolphthalein, and poured in a thin stream into a boiling mixture of 200 ccs. of 10% solution of lead acetate and 100 ccs. of water in a wide-necked ½ litre Erlenmeyer flask. The flask is shaken continuously during the addition. It is then cooled under flowing water with shaking. In this way all the lead soap sticks to the walls and the bottom of the flask and the liquid becomes clear. The liquid is decanted off and the soap washed three times, each time with 200 ccs. of water at 70°–80°, and then cooled. The drops of water hanging on the soap are removed by filter paper, and about 220 ccs. of freshly distilled ether are poured into the Erlenmeyer flask. By shaking most of the soap is loosened from the walls of the vessel and the whole is then warmed with frequent shaking on the water-bath under a reflux condenser for 20 minutes. After cooling under flowing water for ½ hour the lead salts of the solid fatty acids settle down as a powder. The ethereal solution, containing the lead salts of the liquid fatty acids, is poured into a separating funnel, washed with ether and worked up as in 1.

3. **Farnsteiner's process** (*47*). Proceed as in 1 but precipitate with lead acetate [1] and use benzene instead of ether. Dissolve the lead salt in hot benzene (50 ccs. benzene for 1 gm. of lead salt), cool to 8°–12° and leave for 2 hours. Filter, wash with benzene cooled to 10°, dissolve again in hot benzene, and proceed further in the way described and repeat the solution and precipitation once more.

The benzene solution (kept in an atmosphere of hydrogen) which contains the lead salts of the unsaturated fatty acids

[1] If the lead soaps have been prepared in a flask by hot precipitation they may be washed in the flask. By dipping in boiling water the lead salts may be collected on the bottom, most of the water poured out and the residual moisture carefully removed with rolls of filter paper.

(contaminated with the saturated) is shaken in a separating funnel with an equal volume of 10% HCl, separated from the aqueous layer and washed several times with water to remove the lead chloride. The benzene solution, which now contains the free fatty acids, is filtered if necessary, and the solvent distilled off in a current of hydrogen *in vacuo*. The fatty acids remaining behind are thoroughly dried by keeping *in vacuo* for several hours.

The lead salts of the saturated fatty acids insoluble in benzene are decomposed by warming with 20% HCl. The further procedure is as in the case of the corresponding zinc salts.

Separation of the Saturated from the Unsaturated Fatty Acids

1. **Process of A. Grün and A. Janko** (*49*). The fatty acids are converted into ethyl-esters ; these are treated with bromine, whereby the bromo-derivatives of the esters of the unsaturated fatty acids are formed. From these the esters of the saturated fatty acids are separated by fractional distillation *in vacuo*.

2. **Separation of oleic acid from the saturated fatty acids** according to the process[1] of D. Holde, M. Selim and W. Bleyberg (*50*). (According to the principle of Meigen and Neuberger [*51*].)

One gm. of the mixture of fatty acids (or less, down to 0·3 gm. if the quantities of solvent and precipitating agent are also reduced) is dissolved in about 50 ccs. of 96% (by volume) alcohol, neutralised with alcoholic (96%) KOH (about N/2 to N/10), the solution made up to about 125 ccs. with 96% alcohol and treated at room temperature with 65 ccs. of water and 35 ccs. of (4%) aqueous solution of thallium sulphate. After allowing the precipitate of the thallium salts of the saturated fatty acids to settle at 15°, it is filtered through a

[1] A separation tried according to this method by L. Baechler (*Inaug. Diss. Basel*, 1927, p. 62) gave no satisfactory result.

fluted filter at the same temperature. The precipitate is washed with as little as possible 50% alcohol containing a few drops of thallium sulphate solution. From the precipitate and from the filtrate containing the oleate (from the latter after distilling off the alcohol), the fatty acids are liberated by dilute sulphuric acid, extracted with ether, and so on. The method is not suitable for the separation of *erucic acid* and has not as yet been tested for linolic and linolenic acids.

Separation of the Unsaturated Fatty Acids from one another

They are dissolved in about 10 times their bulk of glacial acetic acid, about double the quantity of ether is added and the mixture placed in ice-water. A solution of 1 part of bromine in 2 parts of glacial acetic acid is allowed to flow into it very slowly from a dropping funnel until the bromine is present in excess. It is then kept at about 5° for 12 hours,[1] the precipitate of linolenic acid hexabromide (there are three linolenic acids known) filtered by suction and washed with a cold mixture of equal parts of glacial acetic acid and ether. Melting point of *α-linolenic acid hexabromide*, 180°–181° C. The filtrate from linolenic acid hexabromide is poured into a large bulk of water, the precipitate is filtered off, washed, dissolved in ether and dried with anhydrous sodium sulphate. The ether is distilled off, the residue dissolved in petroleum ether and the solution kept at 0° for 12 hours. The precipitate formed consists of linolic acid tetrabromide and may be recrystallised from petroleum ether. Melting point of *α-linolic acid tetrabromide*, 114°–115°.

The filtrate from linolic acid tetrabromide contains oleic acid dibromide (contaminated with linolic acid tetrabromide (*52*)).

The acids are regained from the bromides in the following way (*53*) : 1 gm. of acid is heated with 5 gms. of zinc filings and 15 ccs. of 90% alcohol for 4 hours under a reflux condenser.

[1] Stronger cooling may also precipitate the linolic acid tetrabromide.

The clear liquid is decanted, and the alcohol, along with that used for washing the zinc, is for the most part distilled off. The residue, a solution of the zinc salt and ethyl-ester of the acid, is poured into 100 ccs. of water. After the addition of 10 ccs. of dilute H_2SO_4 (1 : 10) the mixture is heated on the water-bath for 20 minutes and then shaken twice with ether in a separating funnel. The ether is distilled off and the residue is heated with 5 ccs. of N/2 alcoholic potash in order to saponify the ester. The residue freed from alcohol is dissolved in water, decomposed again with dilute H_2SO_4 (1 : 10) and shaken out with ether. The ether is dehydrated with Na_2SO_4 and distilled off.

The acids can then be identified from their physical properties and by estimation of their iodine values.

Oleic acid. Solidifying point, 4°. Iodine value, 90·07.

α-Linolic acid. Does not solidify even at – 18°. Iodine value, 181·14.

α-Linolenic acid. Iodine value, 273·8.

Oleic acid is also characterised by the Elaidin reaction (see p. 71).

Another method of identifying several unsaturated acids in the presence of each other depends upon their oxidation with potassium permanganate in alkaline solution and separation of the oxy-acids formed by means of solvents. For details see Hazura, *Monatsch. f. Chemie*, 1887, 147, 156, 260 ; 1888, 180, 198, 469, 941 ; 1889, 190 ; also L. Rosenthaler, *Nachweis organischer Verbindungen*, 2nd edition, p. 312.

The identification of some unsaturated fatty acids in the presence of others may be based, according to H. P. Kaufmann (*53a*), on their variable additive power with iodine and sulphocyanides. With erucic acid, brassidic acid and oleic acid the idiometric and sulphocyanometric iodine values agree with each other, while with linolic acid the latter is exactly half of the former. Sulphocyanogen is added only at the double bond of the linolic acid.

The acids with triple bonds hitherto tested, like stearolic acid and behenolic acid, do not combine with sulphocyanogen.

Separation and Identification of Saturated, Non-volatile [1] Fatty Acids

1. **Separation by fractional crystallisation.** The acids are dissolved in so much alcohol that nothing separates out at 15° and the solution is then kept at 0°. The precipitate thus obtained is filtered off. The filtrate is concentrated a little, cooled again to 0°, and so on until no more precipitates are obtained. The melting points and molecular weights of the precipitates are determined; the fractions with similar properties are combined, and their alcoholic solutions are again treated as before until fractions of uniform composition are obtained.

The melting points and molecular weights of the most important fatty acids of this group are given in the following table:

	M.P.	Mol. Weight.
Lauric acid	43·6°	200·19
Myristic acid	53·8°	228·22
Palmitic acid	62·6°	256·26
Stearic acid	69·3°	284·29
Arachidic acid	77° (75°)	312·32

2. **Separation by fractional precipitation** (*54*). The separation depends upon the addition of an alcoholic solution of magnesium, barium or lead acetate to an alcoholic solution of the fatty acids. The solution added must not be in sufficient quantity to cause complete precipitation. The salts of the acids with higher molecular weights are thus precipitated. The precipitation is repeated with the filtrate. One should precipitate, for example, each time with the thirtieth or fortieth part of magnesium acetate (in alcoholic solution)

[1] The expression "non-volatile," which has been taken from colloquial usage, is not quite free from objection since the acids treated here may also be slightly volatile with steam.

required for complete precipitation. The acetic acid set free should be neutralised with ammonia after each precipitation. The precipitated magnesium salts are decomposed by dilute HCl, and the free acids are then purified by recrystallisation or fresh fractional precipitation until acids are obtained which cannot be resolved into acids of different melting points either by fractional precipitation or by recrystallisation.[1]

3. **Examination for palmitic acid (in presence of stearic acid)** (*55*). One gm. of the mixture is dissolved in 200 ccs. of 94% alcohol, placed in ice and filtered after several hours, preferably through an ice-filter. The filtrate is evaporated to half the bulk, treated with a solution of 0·0301 gm. of magnesium acetate in alcohol (in order to precipitate the residual stearic acid) and kept overnight at room temperature. The fatty acid is precipitated from the filtrate by the addition of water or by further concentration when necessary, and the melting point is determined. If it does not agree with that of palmitic acid the fatty acid is again dissolved in alcohol, treated with an alcoholic solution of 0·01 gm. of magnesium acetate and treated further as before. If necessary the treatment should be repeated again.

4. **Examination for stearic acid** (*56*). A saturated solution of stearic acid is first prepared by dissolving 1·5 gms. of stearic acid in 500 ccs. of alcohol, the solution is kept overnight in ice and then the mother-liquid is decanted ; this is best done by using a glass tube bent twice at right angles, the shorter limb being widened into a small funnel and covered with a fine mesh gauze. A quantity of about 0·5–1 gm. of the solid mixture of fatty acids (5 gms. of liquid fatty acids) is then dissolved in 100 ccs. of the solution of stearic acid, by slight warming if necessary, and kept overnight at 0°, preferably in an ice-chest. Next morning it is shaken gently and allowed

[1] Even then mixtures may result.

to stand quietly for half an hour ; the crystals of stearic acid precipitated are then filtered through gauze as described before.

Instead of an alcoholic solution of stearic acid one may also use a solution in methyl alcohol.

5. **Examination for arachidic acid** (*57*). Twenty gms. of oil are saponified by boiling with 10 ccs. of 40% alcoholic KOH and 30 ccs. of alcohol and then acidified with 50% acetic acid (about 15 ccs. are necessary) after the addition of 60 ccs. of alcohol. To the boiling solution a hot solution of 1·5 gm. of lead acetate in 50 ccs. of alcohol is added. It is allowed to stand overnight and the precipitate is then decomposed by warming with 5% HCl. The fatty acids thus obtained (about 2 gms.) are dissolved in 50 ccs. of 90% alcohol with slight warming. The solution is kept in water at 15° for 30 minutes. If anything crystallises out, the crystals are filtered by suction and recrystallised first from 25 ccs. and then from 12·5 ccs. of 90% alcohol. If the amount of the precipitated crystals is small it is advisable to filter by suction in an Allihn-tube over asbestos, dissolve the residue in ether, and to evaporate the ether. The residue left on evaporation is then recrystallised. Since arachidic acid is not quite insoluble in alcohol (0·022 gm. is dissolved by 100 ccs. at 15°) small quantities of it may escape detection by this method.

Identification of Fatty Acids by the Estimation of the Degree of Volatility

The process depends upon the fact, established by R. K. Dons (*58*), that on distilling volatile fatty acids in steam with a definite amount of water a definite amount of acid, characteristic of each acid, goes over, only the last residues of the acid proving exceptions. The *Degree of Volatility* may be defined, according to A. Heiduschka and K. Luft (*59*), as the number of cubic centimetres of N/20 KOH which are necessary to

neutralise (in the presence of rosolic acid as indicator) the acid which goes over with 100 ccs. of water. The degree of volatility is for :

	cc. N/20 KOH		cc. N/20 KOH
Capric acid -	44·8	Lauric acid -	12·0
Myristic acid	3·2	Palmitic acid	0·6
Stearic acid -	0·2	Arachidic acid	0·04

If a mixture of acids is used the portion which distils over from each acid depends only upon the relative proportions of the individual acids present in the mixture. If the degree of volatility of the acid $A=m$ and that of the acid $B=n$, and if the acids are present in the percentage proportion $a : b$, then the degree of volatility of the mixture, which alters in this case at every distillation, amounts to $\frac{a \,.\, m + b \,.\, n}{100}$.

To carry out the estimation a definite amount of the acid is first dissolved, according to Heiduschka and Luft, in a little alcohol and neutralised with N/10 KOH. The alcohol is removed and the acids treated with so much water that a one per cent. solution is obtained.

To an aliquot part of the solution is added (in order to precipitate the acid) a few drops of dilute sulphuric acid and so much water that the total weight amounts to 125 gms. After the addition of a few pieces of pumice stone 100 ccs. are distilled off in the distillation apparatus used for the estimation of Polenske numbers and the distillate filtered directly out of the condenser through a small filter. The filtered fatty acids are dissolved in warm alcohol, which has been previously used for washing the condenser, and titrated with N/20 alcoholic KOH (with rosolic acid as indicator). The filtered water is poured back into the distillation flask ; 100 ccs. are then again distilled off and the process repeated as long as titratable amounts of acid still pass over.

Determination of the Molecular Weights of Fatty Acids

If the fatty acids can be weighed it is only necessary to dissolve them in neutral alcohol and to titrate with N/2 alcoholic KOH (standardised with oxalic acid), using phenolphthalein as indicator. If the molecular weight is represented by M, the weight of fatty acids by y, the number of cubic centimetres of N/20 KOH required by z, then $M = \frac{20000 \cdot y}{z}$ on the assumption that the acids are monobasic.

If the fatty acids cannot be weighed, as in the case of volatile fatty acids obtained from distillates, the molecular weight can still be determined by knowing the amount of alkali required for neutralisation and the weight of soap formed thereby. In such a case one proceeds as follows, according to W. Arnold (*60*) :

The solutions are exactly titrated after the addition of 2 drops of a 1% solution of phenolphthalein in alcohol and the liquid is transferred to a weighed platinum dish (wine-dish). The flask is washed twice with small quantities of alcohol and the soap solutions and washings dried on the water-bath. The platinum dish is kept in a steam oven for $\frac{3}{4}$ hour, the dry soap is then carefully loosened from the bottom of the dish by means of a horn spatula and rubbed to a fine powder with a glass spatula,[1] avoiding any loss. It is dried again for $2\frac{1}{2}$ hours in a steam oven and the soap weighed after complete cooling. From this one should subtract the weight of phenolphthalein added. This is ascertained by evaporating 100 drops of the solution of phenolphthalein (taken with the same dropper) in a weighed dish and drying in a steam oven for 1 hour.

The molecular weight is then calculated from the following formula : $M = \frac{(s - v \cdot f) \times 20000}{v}$, where M = the molecular weight to be ascertained ; s = the mass of soap obtained by

[1] It is advantageous to weigh the glass spatula together with the dish, keep it in the dish from the start and stir with it during the drying.

weighing less the weight of phenolphthalein added, or the mass of the soap less the mass of the residue from the neutralised alcohol containing phenolphthalein; v = number of ccs. of N/20 alcoholic KOH required for neutralisation; $f = 0{\cdot}0019$, increased by a value obtained by means of a sulphate estimation carried out in the following way. Fifty ccs. of N/20 alcoholic KOH are neutralised with sulphuric acid in a weighed platinum dish, a very slight excess of the latter having no effect, a few drops of phenolphthalein serving as indicator. The contents are carefully dried and the sulphate heated to redness. After cooling the weight of the sulphate is ascertained. For example, if 0·00439 gm. of sulphate has been found for 1 cc. of N/20 KOH, f must be increased by $0{\cdot}00439 - 0{\cdot}00435$ [1] $= 0{\cdot}00004$, thus amounting to 0·00194.

Quantitative Estimations and Calculations

The estimation of the content of fat in a substance is done by extraction with a good fat-solvent, usually with ether or petroleum ether. The extraction is carried out in an extraction apparatus, the Soxhlet type and a few of its modifications having proved the most convenient.

After distilling off the solvent it is dried in a steam-drying oven and weighed every two hours. The weighing is taken as correct when the mass becomes constant or begins to increase again slightly in consequence of the absorption of oxygen by unsaturated compounds.

For the quantitative determination of the individual components of a fat one may employ in part the so-called quantitative reactions and in part also some of the processes which are used in qualitative separation. It must be realised, however, that the latter are not always capable of the accuracy which one is accustomed to associate with quantitative separations.

[1] 0·00435 gm. is the theoretical quantity of sulphate corresponding to 1 c.c. of N/20 KOH.

For the estimation of stearic acid see *Zeitschr. f. analyt. Chemie*, 39, 176, 1900, and *Zeitschr. f. Untersuch. d. Nahrungs- u. Genussmittel*, 6, 22, 1903.

The separation of unsaturated fatty acids from the saturated ones is done by forming the lead salts [1] as in the case of qualitative analysis (see p. 80). If the method of Röse is followed, in which the fatty acids are digested with litharge and ether, producing only the normal salts, the average molecular weight of the liquid fatty acids can be calculated from their lead content. If the iodine value of the total fatty acids is also known the content of each fatty acid in a mixture of three different unsaturated acids can be ascertained.

The direct *estimation of glycerine* is performed either by one of the oxidation methods or by the estimation of the acetyl value or by the isopropyl iodide method. In the oxidation methods one may estimate either the amount of potassium dichromate used or the weight of the oxidation product (carbon dioxide or oxalic acid).

In the method of Zeisel and Fanto the glycerine is converted into isopropyl iodide by heating with hydriodic acid, and this is estimated as silver iodide.

The method of estimation of the non-saponifiable constituents of a fat is contained in the method of preparation.

For carrying out these quantitative methods one may refer to the text-book on fat analysis by Benedict-Ulzer, *Analyse der Fett- und Wachsarten*.

From the saponification value (of the neutral fat) the content of glycerine can be estimated, since

1 gm. KOH = 0·5470 gm. of glycerine.

From the saponification value one may also obtain the content of fatty acid in 1 gm. of fat. This = 1 – saponification

[1] For the separation of oleic acid from the saturated fatty acids the method of Holde, Selim and Bleyberg (see p. 82) can also be used.

value × 0·0002258, and the saponification value of the total fatty acids (T.S.K.)

$$= \frac{\text{saponification value of the fat}}{1 - \text{saponification value of the fat} \times 0{\cdot}0002258}.$$

If the content of volatile, water-soluble, fatty acids in the total fatty acids is designated by x, the volatile insoluble fatty acids by y, the non-volatile fatty acids by z, and the corresponding saponification values by K_1, K_2 and K_3, then

$$x \,.\, K_1 + y \,.\, K_2 + z \,.\, K_3 = 100 \times \text{TSK}$$

K_3 as an example will thus be $= \frac{100 \,.\, \text{TSK} - K_1 x - K_2 y}{z}$ (Arnold) (*61*).

From the ester value and Hehner value one may calculate *the quantity of volatile fatty acids* according to the formula,

100 – 0·02258 × ester value – Hehner value (Dieterle).

If I is the iodine value of the oil, I_1 the iodine value of the liquid portion of the fatty acids, then the percentage content of the liquid fatty acids in the oil $= \frac{100 \times I}{I_1}$ (Farnsteiner) (*62*).

VI

WAXES

The examination of a wax is carried out in the same manner as that of a fat. The quantitative reactions and the examination of the fatty acids are the same as those described for a fat. A few components of waxes are soluble in boiling alcohol and crystallise from it on cooling.

To obtain *the alcohols and the non-saponifiable matter* one may follow with advantage the method originated by A. Leys and described by Grimme (*63*) which permits the estimation of these components and of the fatty acids at the same time.

" Ten gms. of wax are boiled with 25 gms. of alcoholic

KOH [1] and 50 ccs. of benzene for half an hour under a reflux condenser. 50 ccs. of hot water are then introduced through the condenser, boiled for ten minutes more and the source of heat removed. The contents of the flask soon separate into two layers, a lower somewhat turbid layer, the alcoholic solution of soap, and an upper clear yellowish layer, the solution of alcohols and hydrocarbons in benzene. The hot soap solution is drawn up with a syphon, 25 ccs. of alcohol are added to the hot benzene solution, 50 ccs. of boiling water poured in and the whole boiled for a further ten minutes. The flame is then removed, and after the separation of the layers the lower layer is removed and combined with the soap solution. The benzene solution is poured into a weighed, flat, porcelain dish, the flask washed several times with hot benzene and the solvent removed by heating on the water-bath. The mixture of alcohols and hydrocarbons remains behind and is dried and weighed.

"*If the alcohols and hydrocarbons are to be separated* the weighed residue is dissolved in 100 ccs. of amyl alcohol, the solution is put into a tall beaker, 100 ccs. of fuming HCl added, heated to boiling with stirring, and allowed to cool very slowly by leaving it standing in warm water. The wax-alcohols, which have gone into solution, now separate out quantitatively in fine crystals, while the insoluble hydrocarbons collect on the surface and set to a solid cake on cooling. The alcohols are filtered, washed with ice-cold alcohol, dried *in vacuo* over sulphuric acid and weighed; the cake of hydrocarbons is dissolved in ether, dried over calcium chloride, and, the solvent having been removed, dried at 100° C. and weighed.

"The fatty acids present in the combined soap solution are freed from alcohol, liberated with dilute sulphuric acid and dissolved in ether. The ethereal solution is washed with water until free from mineral acids and dried over calcium chloride; the ether is removed by evaporating in a

[1] 45 gms. KOH in one litre of absolute alcohol.

weighed flask, the flask and fatty acids dried at 105° C. and weighed."

For the systematic scientific investigation of the waxes, which frequently form very complex mixtures, the following method (*66*) of Gascard (*64*) and Damoy (*65*) may be used. The wax is again saponified according to Leys' method (see above) and the mixture of alcohols and hydrocarbons left after evaporating the benzene, and also the aqueous-alcoholic solution of soap, are worked out as follows :

The separation of alcohols and hydrocarbons is effected by crystallisation from benzene,[1] in which the alcohols are less soluble than the hydrocarbons. The mixture of alcohols eventually obtained is dissolved in 40 times its weight of benzene and filtered at a definite temperature. This purpose is served by the apparatus of Gascard, which requires no further description as its nature is evident from the figure (Fig. 3). Before use a plug of cotton wool is placed in the separating funnel above the stopcock and a little of the liquid used as solvent is put in the bulb. The solution is then put into the separating funnel, which is warmed beforehand, the upper cork with the leading tube attached is inserted, and the whole placed in a thermostat or in a hot chamber which is kept at the desired temperature. In this way four successive filtrations are carried out at four different temperatures, say 40°, 30°, 20° and room temperature. On each filtration some crystals remain in the separating funnel, and the filtrate which runs off them is subjected to filtration at the next lower temperature. The last filtrate is distilled and forms one fraction. Besides benzene 95% alcohol may also be used as solvent.

Fig. 3. Gascard's Apparatus.

[1] The *benzene* to be used for these investigations should be *freed from thiophene* by warming with aluminium chloride.

The fractions obtained are then converted into acetic acid esters,[1] or less often into oxalic acid esters[2]; in the former case the solution in acetone, and in the latter case the solution in chloroform, is subjected to Gascard's fractionation.

For *the analysis of alcohols* the conversion into the iodo-compounds[3] has also proved useful. In the case of alcohols of such high molecular weight as those occurring in beeswax, neither the elementary analysis of the alcohols and their acetic acid esters nor the determination of their molecular weights suffices to distinguish them from their nearest homologues, since the differences between the values fall within the limits of error. As an example the C and H values of the alcohols $C_{27}H_{56}O$ and $C_{26}H_{54}O$, as also those of their acetic acid esters $C_{29}H_{58}O_2$ and $C_{28}H_{56}O_2$, may be quoted :

	% C.	% H.		% C.	% H.
$C_{27}H_{56}O$ -	81·82	14·14	$C_{29}H_{58}O_2$	79·37	13·33
$C_{26}H_{54}O$ -	81·67	14·14	$C_{28}H_{56}O_2$	79·16	13·30

A sufficient distinction is obtained on the other hand by estimating the iodine (according to the method of Baubigny and Chavanne or by the lime method) in the iodo-compounds and finding the saponification values of the acetic acid esters, especially when the amount taken for estimation is not too small (about 2 gms.).

[1] To prepare the acetic acid ester, the alcohol is either heated with acetic anhydride or a current of HCl is passed through a solution of it in glacial acetic acid. It is then poured into luke-warm water, warmed, washed and the cake obtained is dried.

[2] To prepare the oxalic acid ester, the alcohol is heated with double its weight of pure anhydrous oxalic acid in an oil-bath for six hours at 140°. The cake obtained is washed, dried as in (1) and extracted in a Soxhlet with 95% alcohol. The ester remains undissolved.

[3] The preparation of iodo-compounds is accomplished by dissolving 3 molecules of alcohol, 3·6 atoms of iodine and 1·1 molecule of phosphorus in carbon disulphide. The latter is removed in a steam oven and the residue heated for several hours in a bath of $CaCl_2$ at 110°–120° C. Crystals of iodine which sublime on the neck are removed. The reaction product is washed with warm water to decompose the excess of PI_3, dried *in vacuo* and dissolved in alcohol or benzene and ether.

To be more certain it is recommended that the alcohols be oxidised with chromic acid to the corresponding acids.[1] These are purified by conversion into their potassium salts and washing with alcohol or benzene. (Cf. later under the preparation and identification of acids.)

The *hydrocarbons* which are found dissolved in the benzene, together with residues of alcohols and other substances in the above process, are treated first with oxalic acid (see p. 95, note 2). If the reaction product is extracted with alcohol only the hydrocarbons go into solution, and these can be further purified by crystallisation from alcohol, especially if the first precipitated portions be rejected.

The *acids*, which are found in the aqueous-alcoholic layer in the form of potassium salts, are first changed into calcium salts by treating the solution with a large excess of an aqueous solution of calcium chloride. The solution is filtered, the precipitate washed and dried and extracted three times with a large volume of 95% alcohol or ether. The precipitate is dried, powdered and sifted after each extraction. The calcium precipitate, thus freed from impurities, is introduced in small portions into five times its bulk of boiling 10% HCl and after the last addition boiled for a further 10 minutes. The whole is then poured into a larger bulk of water and warmed until the acids collect together as a cake. This is melted again upon warm water, washed, after cooling, with lukewarm water until the washings no longer react acid, then dried in a desiccator and dissolved in 50 times its bulk of 95% alcohol. It is filtered on a steam filter, and the precipitate which separates on cooling is treated according to the method of filtration at definite temperatures.

A distinction between the nearest homologues by means of elementary analysis or by freezing-point determinations is also

[1] For example, 2·5 gms. of ceryl alcohol are introduced in small portions into a suspension of 2·5 gms. of finely powdered potassium dichromate in 50 ccs. of glacial acetic acid. If the dichromate is thrown into the boiling glacial acetic acid solution of the alcohol it also forms the ceryl ester of cerotic acid.

not possible in the case of the acids any more than with the alcohols for the same reason. On the other hand, titrimetric determinations of molecular weights and determinations of the silver contents of the silver salts [1] give good results.

Similar to waxes in their outer characteristics are the *Etholides*, which are obtained by boiling conifer needles with 80% alcohol. Since their constitution can be expressed, according to Bougault and Bourdier (*67*), by the following formula,

$$R.CHOH.(CH_2)n.COO.\underset{\displaystyle R}{\underset{|}{CH}}.(CH_2)m.COO.\underset{\displaystyle R}{\underset{|}{CH}}.(CH_2)p\ldots COOH,$$

they yield only oxy-acids on saponification.

VII

LECITHINS (PHOSPHATIDES)

In connection with the fats and waxes, the lecithins, which are frequently found in plants and at times in seeds, should also be described. They only swell up in water, and are found in the extracts prepared with petroleum ether, ether and alcohol. Lecithin is also found in the latter solvent even after exhaustion with one of the other solvents, since lecithin-containing components are present in plants which split off lecithin on boiling with alcohol. To prepare lecithin, the material is extracted first with ether and then with alcohol at about 60°, the alcoholic extract is evaporated at 40°–50° and the residue is treated with cold ether. The ethereal solution is shaken out with water and any emulsion formed removed with common salt. The ether is evaporated off, the residue taken up with absolute alcohol and the lecithin is precipitated by cooling. It is dried over sulphuric acid.

In the case of materials rich in lecithin the lecithin can be first prepared according to one of the technical methods and

[1] To prepare the silver salt, the alcoholic solution of the potassium salts is treated with silver-nitrate. If the potassium salts are not sufficiently soluble, one may start from the free acids and treat with ammonia.

then purified. In the technique for *the preparation of lecithin from Chinese yolk of egg* the material is either boiled with methyl alcohol and evaporated *in vacuo*, or boiled with alcohol, concentrated, and then precipitated with acetone after separating off the oil.

According to a patented process (*68*) for the purification of lecithin from stearines and fats an aqueous solution of sodium cholate is stirred with lecithin until it is dissolved. Alcohol and finally ether are then added until turbid. By the careful addition of further ether almost the whole of the double compound is obtained as long, fine needles. These are extracted with ether and dissolved in water. The aqueous solution is covered with a layer of ether and treated with 10% HCl. After shaking, the ether which is decanted off is washed with a very dilute solution of sodium carbonate to remove gallic acids and then distilled off.

Alternatively a 50% solution of sodium cholate is rubbed with lecithin until a homogeneous fluid is obtained, shaken twice with ether or benzene in order to remove the impurities (fats and sterols), and the aqueous solution is evaporated *in vacuo*. From the solid residue the lecithin is dissolved out by ether and recovered by evaporation of the ether.

To transform the lecithin into a crystalline double salt, salts of other gall acids (alkali salts of desoxycholic acid, glycocholic acid, apocholic acid) may be used.

For the method of obtaining a crystalline lecithin by strongly cooling an ethereal solution which has been previously treated in a suitable manner see H. H. Escher, *Helv. Chim. acta*, 8, 686, 1925.

To prepare *lecithin from animal organs*, Levene and Rolf (*68a*) utilised the property of lecithin of being precipitated completely out of an alcoholic or acetone solution by means of a saturated solution of cadmium chloride in methyl alcohol. The cadmium compounds are separated from most of the kephaline by repeated extraction with ether, then suspended in four times their bulk of chloroform and treated with a 25% solution of ammonia in methyl

alcohol until no more precipitate is obtained. The lecithin solution is dried *in vacuo* at 35°–40°, the residue is dissolved in ether, the solution evaporated and the process repeated several times. It is finally dissolved in absolute alcohol, when the kephaline remains undissolved. To remove the ammonia, the solution of 50 gms. of lecithin in 50 ccs. of ether is shaken with the same volume of 10% acetic acid and the emulsion poured into 500 ccs. of acetone.

The ether-alcoholic solution of lecithin gives a yellowish white precipitate with alcoholic platinum chloride, which is soluble in ether. On saponification with baryta-water lecithin is hydrolysed to choline or other bases, glycero-phosphoric acid and fatty acids.

The quantitative estimation of lecithin is generally performed by estimating the phosphoric acid obtained on oxidation.

VIII

ESSENTIAL OILS

The preparation of essential oils is generally done by distillation of the powdered material with water or steam. The oil is separated from the distilled water by means of a Florentine flask, or a separating funnel or some similar arrangement. A portion of the oil dissolved in the water can be separated by the addition of common salt, and then by a Florentine flask, or shaken out with ether (water-oil). The various methods by which manufacturers prepare oils and perfumes which are sensitive to higher temperatures and also to steam-distillation need hardly be considered by the phytochemist. These methods are: the mechanical breaking up or pressing out of the secretory cells, maceration (preparation of pomades and their extraction with alcohol), extraction with petroleum ether and the taking up of the extract with alcohol, and enfleurage, in which the perfume is taken up from the fat used for removing the perfume from the scented material. The scent can be extracted again from the fat by means of alcohol.

The most important *physical properties* of the crude oil are first determined; specific gravity, optical behaviour and solubility. A few deductions about the constituents of the oil can be drawn from the specific gravity. A specific gravity below 0·9 shows a high percentage of terpenes or aliphatic compounds, while one above 0·9 suggests the presence of compounds containing oxygen, nitrogen or sulphur or benzene derivatives. If larger amounts of the substances mentioned, especially nitriles, sulphides and thiocyanates are present, the specific gravity may be greater than one.

For *preliminary examination* the following procedure may be adopted:

1. Determination of the reaction. Acid reaction indicates the presence of free acids or phenol-like bodies.

2. Examination for nitrogen (see p. 17). A longer tube should be used.

3. Examination for sulphur. The substance is heated with fuming HNO_3 in a sealed tube in a bomb furnace, and after dilution with water the sulphuric acid formed is tested with barium chloride.

4. Test for aldehydes with an ammoniacal solution of silver nitrate. Reduction takes place (often with the formation of a mirror).

5. Aldehydes and ketones are also recognised by their behaviour with phenyl hydrazine, semicarbazide and hydroxylamine, with which they combine as a rule to form crystalline compounds.

6. Cooling with a freezing mixture. Many oils yield crystalline precipitates in the cold.

7. Elementary analysis. This gives information concerning the presence of compounds containing oxygen.

8. As with fats a few of the reactions used for quantitative estimations also furnish information on the qualitative composition.

(*a*) *Ester value*. If the oil is warmed with alcoholic KOH

(best, N/2 alkali) for about one hour on the steam bath and titrated back with H_2SO_4 the consumption of KOH shows the presence of an ester, provided lactones, free acids and aromatic aldehydes are either absent or have been removed.

(*b*) If free acids or phenols are present the *acid-value* should be determined by titration with cold alcoholic KOH, and the *saponification value* should also be found (like the ester value). The difference between saponification value and acid value corresponds to the ester value.

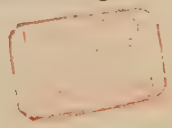
(*c*) A positive result in a *methoxyl determination* by Ziesel's method shows the presence of ether-like compounds. The oil is heated with hydriodic acid, and the iodo-alkyl that may be formed is passed, after suitable purification, through an alcoholic solution of silver nitrate, silver iodide being precipitated.

(*d*) The *estimation of carbonyl* is carried out when aldehydes or ketones are present. The oil is warmed with phenyl hydrazine, the hydrazone formed is filtered, and the excess of phenyl hydrazine is oxidised by boiling with an alkaline copper solution. The nitrogen evolved thereby is collected and measured.

(*e*) *Acetyl determination.* The oil is boiled with acetic anhydride and dry sodium acetate, precipitated by the addition of water, the excess of acid is removed by a solution of sodium carbonate, the excess of soda is removed by water, the oil is dried by anhydrous sodium sulphate and the saponification value is determined. If the saponification value is higher than that of oil not treated in this way then the oil contains alcohols (or phenols or possibly oxy-acids).

If the reactions 1, 4, 5 and 6 are positive the substance is treated accordingly. By cooling, the substances which are precipitated by cold are separated. Acids are extracted by shaking with weak solutions of alkali carbonates, phenols by caustic alkalis.[1] From the alkaline solution the phenol is

[1] Cases where phenols will not combine with aqueous alkalies are exceptional. According to Claisen in such cases a liquor of the following



separated either by the addition of dilute sulphuric acid or by passing CO_2 into the warmed liquid for a long time. The phenols set free are either extracted directly with ether or distilled in steam and obtained by shaking out the distillate with ether.

Aldehydes and ketones, $R.CO.CH_3$, combine with sodium bisulphite when shaken with a concentrated solution. From the compounds thus obtained the corresponding substances may be regained by warming with dilute acids. The compounds of aldehydes and ketones with acid sodium sulphite are also decomposed by alkalies. From the semicarbazides also, which are obtained by treating a concentrated aqueous solution of semicarbazone hydrochloride with an alcoholic solution of potassium acetate and the ketone, with heat if necessary, the ketones may be regenerated by means of acids.

After separating these substances one should attempt to separate as far as possible the remaining components of the essential oil from one another by fractional distillation [1] either at atmospheric or at reduced pressure. The fractionation is also frequently carried out in a current of steam.

The *boiling point* of the fractions often furnishes information about the composition. Thus, the terpenes of the formula $C_{10}H_{16}$ boil between 150° and 190°, the sesquiterpenes about 100° higher, the diterpenes still higher.

The comparison of the elementary composition of the separate fractions with the crude oil furnishes much information, for example, on the quantity of oxygen-containing components in the fractions.

The fractions are examined for the components in the same way as the crude oil and worked accordingly.

composition may be used : 350 gms. of caustic potash, 400 gms. water, methyl alcohol, to one litre. In the decomposition of phenol-potassium compounds by means of carbon dioxide one should remember that instead of the free phenols their acid-alkali compounds may frequently be formed in this way (H. Meyer) (*69*).

[1] In fractionating it is advantageous to use fractionating columns like those constructed by Linnemann, Wurtz, Hempel and others.

Alcohols are distinguished by transformation into esters, especially those of benzoic acid, phthalic acid and succinic acid. Primary alcohols can be combined with anhydrous calcium chloride and the alcoholate again decomposed by water. The phenyl urethanes are also suitable compounds for the separation of alcohols and are formed by the action of phenyl isocyanate upon the alcohols.

To distinguish the *phenols*, the esters, urethanes and phenyl urethanes, etc., are utilised.

According to J. Herzog (*70*), diphenyl urethane is specially suitable for the determination of phenols. The α-naphthyl urethanes also generally crystallise well.

On the preparation of the esters of carbaminic acid and allophanic acid see Gattermann, *Annal. d. Chem.*, 244, 43, 1888 ; on the transformation of phenols into aryl glycolic acids, see F. Schütz, W. Buschmann, and H. Wissebach, *Ber. deutsch. chem. Gesellsch.*, 56, 1967, 1923.

On the use of *p*-nitrobenzyl ether for the identification of phenols see E. Emmet Reid, *Jour. Amer. Chem. Soc.*, 39, 304, 1917 ; 42, 615, 1920, and *Chem. Zentralbl.*, I, 141, 1918, and III, 84, 1920.

The esters present may be saponified and the products of saponification identified.

If the fractions, consisting chiefly of hydrocarbons, contain small amounts of oxygenated compounds, they are distilled again after the addition of metallic potassium or sodium.

The *hydrocarbons* which occur most frequently in essential oils are the *terpenes*, whose separation and identification depend mostly upon the methods discovered and worked out by Wallach. These consist in the preparation of crystalline products from the fractions containing terpenes. The most important of these *terpene derivatives* are :

1. **Halogen acid addition products** (*71*). These are prepared by adding a solution of the hydrocarbon in glacial acetic acid to a solution of the halogen acid in the same solvent and

precipitating the addition compound by pouring into ice-water. If the product is treated in glacial acetic acid solution with anhydrous sodium acetate (*72*) the hydrocarbon is reformed.

2. **The bromide.** The bromide is formed when bromine is allowed to be absorbed by a well-cooled solution of the terpene in ether-alcohol or glacial acetic acid (*73*).

3. **The nitrosite** is obtained by shaking the fractions containing terpenes with a solution of sodium nitrite and acetic acid.

4. **The nitroso-chloride** (*74*). This is obtained by shaking a mixture of terpene and amyl-nitrite with concentrated HCl, keeping the mixture cold and then adding a little alcohol or glacial acetic acid.

5. **Nitrolamine** (*75*) is obtained from the nitroso-chloride by heating it with the alcoholic solution of a suitable organic base upon the water-bath.

In particular cases the terpenes may be identified by *colour reactions*. For example, an intensely blue liquid is obtained by adding a drop of concentrated H_2SO_4 to a solution of a little sylvestrene in acetic anhydride.

C. Mann (*76*) has worked out a method for the *estimation of essential oils in drugs*. His directions are as follows: " 20 gms. of the powdered aromatic substance are mixed with half their mass of pumice and distilled. (For the apparatus necessary for this see *Arch. d. Pharmazie*, 1902, p. 152.) The distillate is salted out, treated with 50 ccs. of rhigolene, shaken well and the volume of rhigolene made up to the original volume. Then 25 ccs. corresponding to 10 gms. of substance are pipetted out and evaporated in a weighing glass. The mass of essential oil thus obtained multiplied by 10 gives the percentage content."

According to Reich (*77*) it is better to transfer the essential oil, dissolved in ether, rhigolene, or pentane, to a Mann's

weighing flask, evaporate off most of the solvent by passing a current of dry air, put in a suitable amount of isopropyl chloride, hold a hot copper-wire gauze in the issuing vapour and continue the current of air until the green halogen-copper flame disappears.

Griebel's Process (*78*). Ten gms. of the powdered aromatic substance (5 gms. of cloves) are treated with 300 ccs. of water in a flat-bottomed 1-litre flask and submitted to distillation after the addition of a few pieces of pumice stone. A doubly-bent distillation tube is used and a vertical condenser (length of the condenser 55 cms., and of the jacket 22 cms.).

The heating is done on wire gauze with a powerful Bunsen burner. An Erlenmeyer flask or a separating funnel, which is marked at 150 ccs. and at 200 ccs., is used as a receiver.

As soon as 150 ccs. have distilled over the flame is removed, the powder adhering to the sides of the flask detached by shaking and another 50 ccs. are distilled off. If turbidity is still observed in the condenser through the separation of essential oils the cooling is stopped temporarily. The distillate is treated with 60 gms. of common salt in the separating funnel and shaken twice, each time with 20 ccs. of pentane. The united extracts are kept for a few minutes in order to allow the salt solution to settle out. The extracts are then transferred to a weighed, wide-necked 100 c.c. Erlenmeyer flask, the pentane carefully evaporated over a moderately heated water-bath, the residual solvent removed with a rubber blower and the flask left in a desiccator for 30 minutes before weighing. It is then weighed. A confirmatory weighing after a further fifteen minutes should show a decrease in weight amounting to only a few milligrams, otherwise weighing should again be repeated after another fifteen minutes. Before estimation the pentane should be tested for its complete volatility.

On the process of O. Dafert see *Zeitschr. f. Landwirtschaftl. Versuchswesen in Deutsch-Österreich*, 26, 105; *Chem. Zentralbl.*, I, 2642, 1924.

On the estimation of essential oils see also W. Peyer and F. Diepenbrock, *Apotheker-Ztg.*, 1926, Nr. 16/17, and *Jahresber. der Caesar and Loretz, A. G.*, 1927, p. 203.

The preparation of *volatile substances*, like those that occur for example in the family of the Ranunculaceae, is similar to that of essential oils. They are distilled in the same way in steam (*79*) and the isolation is attempted by shaking out the distillate.

IX

RESINS

If a resin is to be investigated [1] it should be examined under the microscope in a coarsely powdered state (if it can be powdered) or in the form of a thin smear if soft or semi-soft. It should thus be first ascertained whether it is wholly or partly crystalline, and in the latter case it should also be determined (with the microscope) whether by means of a suitable solvent the crystalline portion can be separated from the amorphous. The presence of easily saponifiable substances may often be detected by treating with a saturated alcoholic solution of KOH or NaOH (see p. 69, note 3) and examining under the microscope in the same way.

The smell and taste of the resin show whether any essential oils and bitter substances are present.

By microsublimation (see p. 18) it can be ascertained whether the resin contains any substance which sublimes easily.

Tests of solubility are then made. In the case of alcohol it should be specially ascertained whether a substance having the properties of a gum remains behind (see p. 142), as a gum-resin can thus be detected.

The alcoholic extract is mixed with water and tested with blue litmus paper for the presence of an acid. Further

[1] For the success of the investigation the proper choice of material is often decisive. One should use, as far as possible, the clear pieces of the originally colourless resin and remove the coloured crusts.

information is obtained by the estimation of the acid value and the ester value (cf. p. 100).

After these preliminary examinations decomposition methods must be resorted to.

In the case of *gum-resins* the resins should be first separated from the gums by treatment with alcohol or ether, essential oils should be separated by steam distillation and bitter substances dissolved out with water ; acids are shaken out of a solution in a suitable solvent (such as ether) by means of alkalies, aldehydes are removed by sodium bisulphite (see p. 102), and so on with others.

Owing to the varied composition of the resins no method can be given by which every resin can be investigated. The processes which are described below and which have proved useful in some cases may with the necessary alterations be also used in other cases.

Investigation of storax according to W. v. Miller (*80*). The water admixed with the storax contained sodium chloride, cinnamic acid and benzoic acid, and the latter was removed by steam.

Distillation of storax with steam gave *styrol*. The residue from the distillation or the '*refined storax*,' strained through a piece of cloth over the water bath, was extracted for two days with cold dilute NaOH and a white powdery residue was left behind.

A current of carbon dioxide was passed through the alkaline solution. The precipitate of '*Storesin*' (see below) was filtered off. The filtrate was heated with HCl and filtered hot through cloth. On cooling the liquid cinnamic acid was precipitated. The resin left on the cloth had a vanilla-like smell and was dissolved in ether. The solution was then shaken with a solution of sodium bisulphite in order to obtain the vanillin.

The white powdery residue mentioned above contained *styracin*. This, however, was more easily obtained by ex-

tracting the 'refined storax' with petroleum ether, which extracted the *styracin* (*cinnamyl-cinnamic ester*) as well as phenyl-propyl-cinnamate and ethyl-cinnamate. The resin thoroughly extracted with petroleum ether was taken up with a mixture of ether and petroleum ether. The residue remaining after this was purified by solution in ether and precipitation with petroleum ether. The residue obtained from this solution was dissolved in ether and shaken with a very dilute solution of sodium carbonate in order to remove the free cinnamic acid. The residue obtained from the ethereal solution was saponified with dilute KOH. The saponified solution gave a precipitate with dilute KOH which consisted chiefly of potassium-α-storesin and was decomposed by HCl. A further quantity of storesin was obtained by passing CO_2 through the alkaline filtrate.

A separation of α- and β-storesin was attained by treating the mixture with 1 : 1000 KOH. The first extracts contained fairly pure β-storesin, the next ones a mixture of α- and β-storesin, and the last pure α-storesin.

Investigation of Siam-benzoin according to F. Reinitzer (*81*). The Siam-benzoin was fractionally dissolved in ether, and the later extracts (the first solutions were worked out separately) gave crystalline aggregates of lubanol benzoate by the addition of petroleum ether until a turbidity appeared on cooling to – 3° to – 6° C. The lubanol benzoate was obtained pure by repeating the precipitating process.

The mother-liquors, which gave no more crystals on prolonged keeping, were distilled carefully on the water bath, first at 25°–35°, then at 35°–45°, until the liquid in the distillation flask was so concentrated that it was highly turbid owing to the separation of white amorphous resin. On keeping in the cold a few crystals of lubanol benzoate were obtained along with finer sandy crystals of siaresinolic acid. By decantation and washing with ether-petroleum ether the amorphous resin could be removed. It was filtered and purified by solution

in ether and precipitation with petroleum ether. The crystals of lubanol benzoate and siaresinolic acid could be separated from each other by cold ether which dissolved the siaresinolic acid only slowly and with difficulty, leaving the unattacked sandy crystals behind. The last mother-liquors gave crystals of benzoic acid on evaporation.

The sodium salt of siaresinolic acid could also be obtained by treating the benzoin with 4–5% NaOH, by which it remained undissolved. It could be obtained pure by crystallisation from alcohol.

Investigation of copal. (*a*) According to P. Horrmann (*82*). 500 gms. of copal were shaken for three days with 1500 ccs. of ether. The ether was decanted off and the residue again shaken with ether. The ethereal solution was first shaken with 500 ccs. of a 2% solution of ammonium carbonate, and after separation the latter was poured into a dilute solution of H_2SO_4. The ethereal solution of the resin was shaken as before exhaustively with a 2% solution of Na_2CO_3 with the difference that the soap solution was washed each time with fresh ether in order to dissolve out the essential oil and other substances soluble in ether. From the solution of the resin soaps the acids were precipitated by dilute sulphuric acid.

From the ethereal solution the ether was distilled off and the essential oil removed from the residue by means of steam ; the neutral resinous constituents remained behind.

To purify the crude acids a current of carbon dioxide at low pressure was passed through their solution in 2% Na_2CO_3 until the solution was saturated ; the resin acids were precipitated from the filtrate by HCl. The procedure was repeated with these precipitates until there was no more precipitate with carbon dioxide.

The portion of resin which was found insoluble in ether was dissolved in nearly double the volume of alcohol. After filtering off the mechanical impurities the alcoholic solution was poured drop by drop into boiling water.

(*b*) According to J. Scheiber (*83*). Every 250 gms. of the resin, cut into pieces of the size of beans, were treated with about 1 litre of a 5% solution of caustic potash and then heated in a current of steam. A clear solution of the resin was thus obtained in the course of 1–2 hours, the essential oil collecting in the receiver at the same time.

The dark-brown solution of resin was filtered by percolation and after warming to about 30° acidified with HCl. The precipitate was filtered off and after washing with cold water put into a 5% solution of ammonium carbonate. Altogether 15–25 litres of ammonium carbonate solution were used for the precipitate from 1 kilo of copal. The whole was put into glass flasks and kept for several days with frequent shaking. It was then filtered. From the filtrate the crude acid was precipitated by HCl, washed, pressed between filter papers and dried at a moderate temperature. The crude acid was purified by recrystallising the dry product from a mixture of equal parts of ethyl and methyl alcohol.

Investigation of a tolu balsam according to E. Fourneau and M. Crespo (alcoholysis) (*83a*). 250 gms. of tolu balsam were heated under a reflux for six hours with 500 gms. of 96% alcohol, containing 15 gms. of HCl gas. A very concentrated solution of 20 gms. of sodium carbonate was then added, taking care that the solution remained faintly acid. Steam was then passed, the distillate extracted with ether, the ethereal liquid dried with $CaCl_2$ and the ether distilled off. The residue was distilled at 18 mm. pressure and obtained in four fractions. The first fraction (below 100°) contained tolene and ethyl benzoate, the second fraction (100°–104°) contained the latter, the third fraction (104°–115°) contained a mixture of cinnamic acid and ethyl benzoate, and the fourth (about 145°) contained ethyl cinnamate.

It is clear that the quantitative composition of a resin cannot be ascertained by any of these methods.

For other methods see A. Tschirch, *Harz und Harzbehälter*. Gebr. Bornträger, Berlin, 2nd Edition, 1906.

On the investigation of resins by the use of superheated steam see H. Wislicenus, *Zeitschr. f. angew. Chem.*, 41, 1500, 1927.

X

TANNINS

The preparation of pure tannins is, for various reasons, attended by difficulties which are still in part insurmountable. They are mostly amorphous, of colloidal nature, and give no crystalline compounds. Their solutions often possess the property of keeping substances in solution which are not soluble in the pure solvent. On the other hand, the solubility of tannins is influenced by other substances. To these substances belong in particular other tannins, so that the solubility relations of the pure tannins are quite different from what would be expected from their relations with the solvent. According to K. Freudenberg (*84*) an aqueous solution of a tannin is a supersaturated solution of a crystalline substance which is soluble with difficulty in cold water. A further difficulty is caused by the fact that many tannins are easily decomposed by various physical and chemical actions, and that the decomposition products are, therefore, often very difficult to separate.

Many tannins, *e.g.* quinotannic acid, are very sensitive to oxygen and one should, therefore, work in an oxygen-free atmosphere (N, CO_2) for their preparation.

Again, they are mostly accompanied in plants by enzymes which decompose them. It is, therefore, necessary to sterilise the material by pouring it in small quantities into the boiling liquid with which the substance is to be extracted. The tannins are also attacked by the enzymes of fungi and by bacteria. It is thus necessary either to work aseptically or to protect the aqueous extracts and solutions by an antiseptic (toluene, chloroform).

Moreover, there is often more than one tannin in many

plants and there is no general method applicable for the separation of two tannins from one another.

The *solvents* generally used *for the extraction of tannins* from raw materials are: ether-alcohol, strong or dilute alcohol, water, acetic ether and methyl alcohol.

K. Feist and H. Bestehorn (*85*) extract the drug containing tannin first with benzene, chloroform and ether in succession and then extract the tannin with a mixture of acetone and alcohol.

In using ether-alcohol a mixture of 4 parts of ether and 1 part of alcohol should be taken. The extract is shaken (best in a separating funnel) with a third part (by volume) of water. The water takes up most of the tannin. For purification the aqueous solution is shaken a few times with ether and the solution either evaporated to dryness or purified further. The evaporation must only be done *in vacuo*. The purified tannin may also be concentrated *in vacuo* to the consistency of a syrup instead of evaporating to dryness. The extract is spread in thin layers over plates and finally dried *in vacuo* over H_2SO_4.

If it has been evaporated to dryness the residue is dissolved in a little alcohol or acetic ester and precipitated fractionally with ether. By repeating this process one may now and then succeed in obtaining a pure tannin. A further purification of the tannin obtained in this way may be attained by the methods described below. It may also be precipitated from solution in methyl alcohol by ether. The methods used for the further treatment of the aqueous solution obtained from ether-alcohol may also be used for the aqueous solution of the tannin obtained by extracting the crude material with water.

Other methods of purification are:

(1) Precipitation with common salt, followed by shaking with acetic ether or acetone.

(2) Precipitation as compounds with metals and recovery of the tannins by decomposition of the same.

Löwe (*86*) recommended precipitation with common salt, etc. He dissolves the tannin in a solution not fully saturated with common salt, filters off from the precipitated impurities and then precipitates the tannic acid by the addition of common salt. The operation is repeated, the tannic acid finally dissolved in a mixture of one volume of saturated common salt solution and two volumes of water and shaken out from this with acetic ether. It is not necessary to obtain the tannin from the aqueous solution by evaporation first, but fractional precipitation with common salt may be undertaken directly from the aqueous solution. The following modification of the process may then be used. A small portion of the tannic acid and the impurities that may be present are precipitated from the aqueous solution by the addition of some common salt, filtered, and the solution shaken several times with ether. The ethereal solution is examined specially for gallic acid and similar products of hydrolysis of tannins which are frequently present in the extracts along with tannins. To the aqueous solution common salt is added until saturated and then shaken out with acetic ether. In evaporating the acetic ether solutions the same precautions should be observed as described above for the aqueous solutions.

The compounds of heavy metals used up till now for the precipitation of tannins have mostly been those with neutral or basic lead acetate and copper acetate. The use of acetates, however, is not free from objections. The acetic acid set free during the precipitation may hold a portion (although small) of the tannin-heavy-metal-compound in solution and may alter the still unprecipitated tannic acid. Moreover, it often hiders the subsequent examination. One may, therefore, use *lead hydroxide* or freshly precipitated lead carbonate instead of the lead acetate ; the former, however, attacks the tannin, owing to its basic properties. The best experience I have had has been with *copper carbonate*. Copper hydroxide can also be used for precipitation ; the copper

carbonate is, however, to be preferred because copper hydroxide forms insoluble compounds with certain substances which are not precipitated by the carbonate. The dry (commercial) copper carbonate may also be used for precipitation. The amorphous carbonate, which is obtained by precipitating the cold solution of a copper salt with alkali carbonate, acts more rapidly and completely. The precipitation with copper carbonate may also be done in fractions, at least in three fractions, and the properties of the tannins obtained from these compared with one another.

The precipitates, which are obtained by the action of lead or copper compounds with tannins, are washed, suspended in water, and decomposed either by H_2S or by dilute HCl or H_2SO_4 in insufficient quantity for complete decomposition and shaken out with acetic ether.

K. Freudenberg and G. Uthemann used an aqueous solution of *thallium bicarbonate* in order to precipitate the tannins. The precipitates were decomposed by dilute HCl in a mixture of acetone and water.[1] The thallium chloride is insoluble in this mixture and may be filtered off.

In some cases the method used by E. Fischer (*91*) for the *separation of gallotannic acid and gallic acid* can be employed for the isolation of pure tannins.

1. The tannic acid is stirred with an equal bulk of water, a layer of acetic ether put upon it and treated, during stirring, with sufficient 2*N* NaOH to render the liquid distinctly alkaline to litmus paper (the quantity necessary being first ascertained by a preliminary experiment with a small quantity). The solution is then shaken rapidly and repeatedly with freshly distilled acetic ether, the combined extracts containing the tannic acid are washed three to four times with water, each time with 10 ccs., and then evaporated to the consistency of a syrup under reduced pressure, and so on (E. Fischer).

[1] The account of Freudenberg runs thus: Aqueous acetone (1 : 2 vol.). K. Freudenberg, *Die Chemie der natürlichen Gerbstoffe*, p. 33.

2. A solution of tannic acid in four parts of water is cooled to 0° C. and treated during vigorous stirring with so much *N* KOH in a thin stream that the liquid does not redden blue litmus paper. The precipitate thus obtained (potassium tannate) is filtered by suction, pressed, dissolved in approximately the same volume of hot water as was used for the original tannic acid and precipitated again by cooling to 0°. The filtered and pressed precipitate is transferred to a separating funnel, treated with *N* H_2SO_4 and the tannic acid taken up with acetic ether (Berzelius, E. Fischer).

Ellagic acid was partially separated by Feist and Bestehorn (*87*) from the crude tannin of the oak bark by allowing the ellagic acid to crystallise out of a solution of the tannin in hot pyridine.

To free the tannins from inorganic matter one may best use the method of *electro-osmosis*, for which the apparatus described below (Feist and Bestehorn) may be used.

" The dialyser proper consists of a tube *N* bent like a syphon, the ends of which dip into the beakers *A* and *K* containing the electrodes and are fitted with membranes of parchment paper. At the top of the tube is fused a small tube *R* through which the gases evolved may come out and which also serves to fix the apparatus to a stand. The syphon is filled with the solution through the tube *F* which is fused through the cathode side. This tube reaches almost to the membrane at the bottom and widens at the top into a funnel. The solution can flow out through the overflow tube *U* which is fused to the other arm of the syphon and which reaches likewise almost to the membrane at the bottom. The solution to be dialysed drops gradually under constant pressure from the reservoir *V*, and after complete purification it falls out of the overflow tube into the receiver *S*. By regulating the velocity of the drops it can be so arranged that every portion of the liquid will remain for several hours in the dialyser under the influence of the potential difference set up between the

electrodes *K* and *A*. The flow of the tannin particles in the vessel is adjusted to take place in the same direction as they stream in the electric field, and that of the ions in the opposite direction; the ions are carried from their place of entry to the bottom of the inflow tube directly through the neighbouring membrane into the cathode liquid which is now and then renewed. With the dimensions of the apparatus used (the sketch being one-eighth of the natural size) the substance can be obtained quite free from ash with a velocity of 26 drops per minute (=1 litre in 12 hours)."

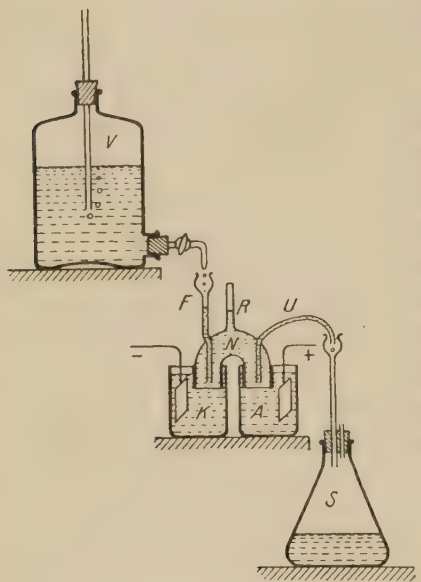


FIG. 4. Apparatus for electro-osmosis.

A purification of tannins is often attained by converting them into their derivatives. Thus K. Feist and H. Bestehorn (*87*) succeeded in purifying the tannins of the oak bark by methylation, since the methylated tannins are more easily soluble in acetone than the methylated impurities. On the separation of tannins and anthocyanidines see p. 68.

The separation of two tannins from one another is seldom effected by fractional precipitation,[1] but better by their different behaviours towards solvents. For example, one

[1] Karrer, Salomon and Peyer (*90*) used *freshly precipitated aluminium hydroxide* for this purpose.

The aluminium hydroxide is prepared from pure $Al_2(SO_4)_3 + 18\ H_2O$: ammonia is added drop by drop to a 10% hot aqueous solution of this salt with constant stirring until the reaction of the liquid is neutral (the quantity of ammonia necessary being determined by a special experiment). The aluminium hydroxide is filtered off, washed well with water and used fresh.

Each precipitate of tannin with alumina is likewise decomposed with the

tannin may be precipitated from its alcoholic solution by ether while the other remains in solution in the mixture of alcohol and ether.

The detection of two tannins in the presence of one another, when a separation cannot be affected, may be accomplished by decomposing one of them. Zölffel (*88*) has in this way demonstrated that two tannins are present in the fruits of Caesalpinia brevifolia and in myrabolons by decomposing one of them by boiling with dilute H_2SO_4. The gallic acid formed by hydrolysis is removed from the filtrate by shaking with ether, the H_2SO_4 is precipitated by baryta, and the tannin isolated by the lead acetate method.

A substance which may be stated to be a tannin should give the following *reactions* :

(1) Precipitate with gelatine and alkaloids.

(2) Blue or green colour with ferric salts.

(3) Brown colour or precipitate with potassium dichromate.

(4) Colour reactions are given, among others, by uranyl acetate (reddish brown), ammonium vanadate (blue or green), ammonium molybdate (with gallic acid, red).

Phloroglucin tannins are reddened with vanillin-HCl. All the tannins isolated should be tested to determine whether they yield sugar on hydrolysis.

Tannins, which are to be analysed, must be completely freed from the organic solvents with which they are in contact, then dissolved in water, and the water removed *in vacuo* and finally in high vacuum (Freudenberg).

Most of the processes developed for the *quantitative estimation of tannins* refer only to gallotannic acid and are therefore not applicable generally. The gravimetric *method of Schröder* (*89*) can be used for all tannins.

Twenty-five gms. of the powdered plant material are first

required quantity of dilute sulphuric acid in the cold and the free tannin should be shaken out when possible (gallotannic acid with acetic ether). The solutions of the tannins are washed repeatedly with water, dried with anhydrous sodium sulphate and then evaporated *in vacuo*.

extracted by boiling for half an hour with 500 ccs. of water and the residue is then fully extracted in a percolation apparatus so that one litre of liquid results.

" 100 ccs. of the tannin solution are evaporated to dryness in a platinum dish on the water-bath, the residue dried at 100° to constant mass and weighed : total amount of soluble matter (G). The residue is then converted into ash and the amount of ash ascertained (A). G – A then gives the amount of soluble organic matter in 100 ccs. of tannin solution (O). 200 ccs. of the tannin solution are then digested for one hour with 10 gms. of hide powder with frequent stirring, the mass pressed out in a linen filter and the filtrate treated again for 24 hours with 4 gms. of hide powder.[1] From the filtrate 100 ccs. are evaporated to dryness on the water bath, the residue dried at 100° to constant mass and weighed. It is then converted into ash and the mass of ash deducted from it. In this way the quantity of non-tannin (N) present in 100 ccs. of tannin solution is obtained. From this one should deduct the mass of the small amount of organic matter dissolved out of the hide powder as determined by a direct experiment under the same conditions. The quantity of the actual amount of tannin present in 100 ccs. of the solution is finally obtained from O – N."

The method of Löwenthal-von Schröder (*93*) may also be used for the estimation of tannin after previously ascertaining the behaviour of the pure tannin with potassium permanganate under the conditions of the process.

If the tannin is found to be a gallotannic acid the process of Ruosz (*93*) may be used.

For the *biological method of testing for and estimating* tannins (by agglutination of blood corpuscles) see R. Wasicky, *Collegium*, 553, 1916 ; 572, 1917 ; *Pharmaz. Post* 50, 785, 1917 ; also W. Brandt, and F. Schlund, *Pharmaz. Ztg.* 69, 597, 1924.

[1] One should see that all the tannins have been removed by testing the filtrate with a tannin reagent (see p. 117) ; if not, more hide powder should be added.

For the estimation of tannins in drugs see also Schulte, *Pharmaz. Ztg.* 67, 497, 1922.

For the estimation of tannins by precipitation with titanic chloride and back titration of the excess by iron alum see S. Krishna and N. Ram, *Ber. deutsch. chem. Gesellsch.* 61, 771, 1928.

For the separation of tannins from vegetable acids see p. 121.

XI

PHLOBAPHENES

Closely related to the tannins are the phlobaphenes, the decomposition products of tannins. They are themselves insoluble in water, but may remain in aqueous solution in the presence of other substances, especially tannins. If the aqueous solution is evaporated to dryness (*in vacuo !*) and the residue taken up with absolute alcohol the phlobaphenes remain undissolved. They may be purified by solution in alkalies and precipitation with acids. They are also tested for glucosidic properties.

XII

ORGANIC ACIDS

The facts observed during the course of the preliminary examination should also furnish the basis for the preparation of organic acids. One should be in a position to state whether the acids are present in the free state or combined, and whether, in the latter case, they occur as salts of alkalies or of alkaline earth metals ; also, whether they can be easily separated in any particular stage of the investigation, or whether they will have to be extracted in a special manner. The latter should be done only when the acids form a preponderating and characteristic component of the drug.

Most of the acids go into solution when the drug is boiled with water containing HCl or even with acidified alcohol.

For further investigation (specially for acids already known) the acids should be converted into their sodium salts by neutralisation with sodium carbonate after the alcohol has been removed by evaporation. If there is a precipitate on the addition of sodium carbonate to the aqueous solution (*e.g.* of $CaCO_3$) the solution is boiled for 15 minutes (or longer with larger amounts) with excess of sodium carbonate solution and then neutralised with acetic acid. Many organic acids can be precipitated from this solution by lead acetate and may be regained by decomposing the lead compounds with H_2S. Many different methods have been worked out for the separation of organic acids which occur frequently, most of them depending upon the behaviour of the acids with Pb, Ca, or Ba salts. If an aqueous solution of the neutral alkali salts has been obtained by the above process one may first precipitate,[1] according to E. Fleischer (*94*), *tartaric acid*, *citric acid*, *malic acid* and *oxalic acid* by means of lead acetate. The precipitate, which may contain the lead salts of H_2SO_4, HCl and H_3PO_4 in addition to those of the above organic acids, is washed with 50% alcohol[2] and then treated with ammonia. The filtrate contains *tartaric acid*, *citric acid* and *malic acid*. It is digested with ammonium sulphide and acidified with acetic acid. The liquid filtered from PbS contains the free acids. The liquid is concentrated if necessary, an aqueous solution of potassium acetate is added in sufficient amount and the *tartaric acid* is precipitated as cream of tartar by the addition of 95% alcohol (double the volume of the aqueous solution), the precipitation being accelerated by vigorous stirring. After one hour the liquid is poured off and the

[1] If the extraction has been done by water the mucin-like substances that may be present are first precipitated by the addition of an equal volume of alcohol.

[2] According to F. Auerbach and H. Weber (*95*), the *lead salts* of some vegetable acids are fairly soluble in 50% alcohol. One litre of 50% alcohol dissolves 14 mg. citrate, 17 mg. succinate, 26 mg. malate and 1·892 gms. of benzoate. The tartrate is insoluble and can be freed from the other lead salts mentioned by means of 50% alcohol.

residual cream of tartar washed with a mixture of 2 parts of alcohol and 1 part of water. If calcium chloride, ammonia and some alcohol are added to the liquid filtered from cream of tartar the calcium salts of *malic acid* and *citric acid* are precipitated. By washing with hot lime-water the calcium malate can be separated from the calcium citrate which remains undissolved.

The portion of the lead precipitate insoluble in ammonia is decomposed by H_2S. To the free acids one should either add a sufficient quantity of Na acetate or neutralise with sodium carbonate and acidify with acetic acid. The *oxalic acid* is then precipitated as calcium oxalate by a saturated solution of calcium sulphate.

The free acids are liberated from all the precipitates obtained, and examined very closely not only by specific reactions but also as far as possible by elementary analysis and determinations of molecular weights. It is only by these means that it is possible to detect the presence of new organic acids giving the same precipitation reactions as known acids when both are present together. To liberate the organic acids from their salts the lead salts are treated with H_2S, and the other salts are either treated with dilute sulphuric acid or first converted into the lead salts. Thus cream of tartar may be dissolved in hot water, and the tartaric acid again precipitated with lead acetate, and so on.

If Fleischer's process is followed, the *tannins*, which are precipitated simultaneously by lead acetate, should be removed before the precipitation with lead acetate. This may be accomplished by shaking the solution with acetic ether and by treatment with hide powder, proteins or a solution of glue. They may also be removed by precipitation with alkaloids or with antipyrin. Gallic acid, which may occur as the hydrolytic product of tannins (specially after treatment with acids), is also removed by shaking with acetic ether.

To remove tannins and many other interfering substances

one may also use the " washed alumina " of H. Wislicenus (available from E. Merck, Darmstadt).

To isolate *succinic acid* the method of v. d. Heide and Steiner (*96*), worked out for the estimation of succinic acid in wine, may be used as follows : 50 ccs. of liquid freed from alcohol are mixed with 1 cc. of 10% $BaCl_2$ solution, and, after the addition of one drop of alcoholic solution of phenolphthalein, treated with finely powdered barium hydroxide in small portions until there is a red colour. The liquid is concentrated during that time to exactly 20 ccs. as nearly as possible. After cooling, 85 ccs. of 96% alcohol are added during vigorous stirring. The barium salts of *succinic acid*, *tartaric acid* and *malic acid* are thereby precipitated along with other constituents while those of lactic acid and acetic acid remain in solution. After at least two hours' standing the precipitate is filtered off and washed a few times with 80% alcohol. The precipitate is transferred with hot water into an evaporating basin. The contents of the basin are concentrated over a boiling-water bath in order to remove the alcohol completely, and then treated, during further heating, with 5% $KMnO_4$ solution, 3–5 ccs. at a time, until the red colour persists for 5 minutes. Then 5 ccs. of the permanganate solution are added and allowed to act for 15 minutes more. If the red colour disappears again the last operation is repeated. If the oxidation is complete the excess of potassium permanganate is destroyed by sulphurous acid. After the disappearance of the red colour the solution is carefully acidified with 25% H_2SO_4 and the addition of sulphurous acid is continued until the MnO_2 is also dissolved. It is then evaporated down to 30 ccs., mixed with 40% H_2SO_4 until the liquid contains about 10% of free H_2SO_4, and shaken out with ether, preferably in a perforation apparatus. Only the succinic acid goes into the ether (the tartaric and malic acids being decomposed by the treatment with permanganate) and may be obtained by evaporating off the ether.

Among the reactions for identification which can be used

with the isolated acids or the neutral alkali salts obtained by neutralising them with alkali carbonate the following may be emphasised.

1. **For citric acid.**

(*a*) With lime water it gives a precipitate first on heating which dissolves again on cooling unless the heating is continued too long.

(*b*) A small quantity of the acid is dissolved in a little water, a few drops of *N*/10 $KMnO_4$ are added and carefully warmed (up to about 30°). If the liquid is coloured brown, or if there is a precipitate of hydrated manganese dioxide, a few drops of ammonium oxalate solution are added and then about 10 ccs. of 10% H_2SO_4. If a few drops of bromine water are added to the now decolourised clear liquid a turbidity due to pentabromacetone is observed if citric acid is present (*Stahr's Reaction*) (*97*).

(*c*) If the solution of the acid is treated with about one-twentieth the volume of a solution of mercuric oxide in sulphuric acid,[1] heated to boiling, and a few drops of *N*/10 $KMnO_4$ are added a white precipitate is formed if citric acid is present (or any ketonic acid). *Denigès' Reaction* (*98*).

Microchemistry : (1) $\frac{1}{10}$ mg. of citric acid and 2 mg. vanillin are taken in a watch glass. A micro-drop of concentrated sulphuric acid is added to it without mixing. The watch glass is then placed over a beaker containing boiling water for 10 minutes. After 3 minutes the mixture becomes partially brownish violet, and after 10 minutes it shows a violet shade when held against light. If it is now allowed to cool and 3 micro-drops of water added the colour changes to green. If 7 micro-drops of ammonia are then added the colour turns to rust red (*99*).

(2) See page 130.

[1] 5 gms. of HgO are dissolved in 20 ccs. of concentrated H_2SO_4 and diluted with 100 ccs. of water.

2. **For tartaric acid:**

(*a*) The precipitate formed in a solution of the neutral salt with $AgNO_3$ is blackened on heating.

(*b*) If tartaric acid is added to a mixture of resorcin and concentrated H_2SO_4, heated previously to 125°–130°, a reddish colour is produced (*Mohler's Reaction*) (*100*).

(*c*) If the solution of a neutral tartrate is heated and a solution of $FeCl_3$ poured in drop by drop there is a yellow precipitate (*101*).

Microchemistry: (1) One micro-drop of concentrated sulphuric acid is heated on a crucible lid with a crystal of resorcin of half the size of a pin's head ($\frac{1}{4}$ mg.) until the resorcin colours the drop to a reddish or bluish tint. A minute particle of the tartaric acid or a tartrate is then added. Moderate heat with a micro-flame gives a deep violet colour (*99*, *100*).

(2) See p. 130.

3. **For malic acid:**

(*a*) If a tenth part of a 5% solution of mercuric acetate and 1 cc. of acetic acid are added to its solution and filtered and heated to boiling, and a 2% solution of $KMnO_4$ is then allowed to flow in drop by drop, a white precipitate of the mercury compound of oxalacetic acid is formed (*Denigès' Reaction*) (*102*).

(*b*) Palladium chloride is reduced (*103*) if it is heated to boiling with a neutral or weakly alkaline solution of a malate.

(*c*) On heating malic acid to 150°–200° it is converted into fumaric acid and maleic acid. The crystals of fumaric acid are distinguished by their difficult solubility in water.

Microchemistry: (1) 50 mg. of diazobenzene sulphonic acid are dissolved in 1 cc. of 40% KOH. One micro-drop of this freshly prepared solution is placed upon a crucible lid and a particle of malic acid is dropped into it. The particle becomes surrounded by a characteristic narrow reddish brown ring (*104*).

(2) See p. 130.

4. For oxalic acid:

(*a*) The acid and its neutral salts give a precipitate of ferrous oxalate with ferrous sulphate.

(*b*) Ferric chloride produces a green colour with excess of oxalic acid.

(*c*) When heated with concentrated sulphuric acid oxalic acid is decomposed into CO, CO_2 and H_2O without charring (blackening).

(*d*) With $CaCl_2$ an oxalate gives a precipitate of calcium oxalate which is insoluble in acetic acid.

Microchemistry : (1) 1 mg. of oxalic acid or 10 mg. of oxalate and 2 mg. of resocrin are melted together very carefully on a crucible lid over a micro-flame. After cooling, one micro-drop of concentrated H_2SO_4 is allowed to flow by the side. Repeatedly heating for 3 seconds at 20 second intervals produces a dark blue and then a green colour. On cooling the stain becomes yellow and on warming the green colour returns intensified (*105*).

(2) See p. 130.

5. For succinic acid: Succinic acid melts at 185° and sublimes easily.

(*a*) Succinates give a brown precipitate with ferric salts, a precipitate with $CaCl_2$ only in concentrated solution, but with $Ba(OH)_2$ and $BaCl_2$ also in dilute solution.

(*b*) Ammonium succinate (obtained by evaporating succinic acid and ammonia to dryness) gives, on heating, fumes of pyrrole which colour a match-stick red when moistened with concentrated HCl.

Microchemistry : (1) If a particle of lead acetate is put into the solution, rhombohedral, wedge-shaped or clustered crystals are shortly formed (*106*).

(2) On recrystallisation from a solution of saffranin colourless-violet pleochroic crystals are obtained (*107*).

Identification of Chlorogenic Acid (108)

The plant or a portion of it is boiled with 5–10 times its bulk of 10% H_2SO_4 for at least one hour. The solution is filtered, and, after cooling, shaken twice with an equal volume of ether. The combined ethereal solutions are shaken with one quarter of their volume of water. The ether is then distilled off and the following reactions are carried out with the residue :

(1) A little of the residue is dissolved in a large bulk of water so that a solution of about 1 : 50,000 is obtained. To a portion of the solution 2 drops of dilute $FeCl_3$ solution (1 part of medicinal $FeCl_3$ solution +99 parts of water) and then 2 to 3 drops of 1% sodium carbonate solution are added (avoiding excess). A persistent blue colour is obtained.

(2) If to another portion of the solution (1 : 50,000) a few drops of 10% NaOH are added, a rose colour is gradually developed, reaching a maximum after 1–2 minutes and becoming yellow on acidification. In less dilute solutions a dark red colour is observed instead of a rose colour.

These reactions arise from the decomposition products of caffeic acid.[1] The

Identification of Caffeic Acid

can be done in the following way :

The dried, powdered plant is boiled with ten times its bulk of 80% alcohol. The solution is filtered hot, the alcohol distilled off, the residue taken up in a volume of boiling water equal to that of the material used, and the still warm liquid is shaken twice with an equal volume of ether. The separated aqueous liquid is diluted with 5–10 parts of water, filtered if necessary, and precipitated with lead acetate. The lead precipitate is filtered and washed, and then decomposed with

[1] Chlorogenic acid is decomposed by hydrolysis into caffeic acid and quinic acid.

cold 10% H_2SO_4. The filtrate, which now contains impure chlorogenic acid, is treated with so much 50% KOH that the liquid contains 10% of free alkali. It is boiled for half an hour, the still hot liquid treated carefully with 33% H_2SO_4 until just acid, and the cooled luke-warm liquid shaken twice with its own volume of ether. The ether is distilled off and the residue taken up with thirty times its weight of boiling water. After adding a little animal charcoal it is filtered, whereupon the caffeic acid crystallises out. It can be purified by re-crystallisation from water. It melts at 209° C. The aqueous solution turns green with ferric chloride ; if sodium carbonate is added to it there is first a blue and then a reddish violet coloration.

Identification of Aconitic Acid (*109*)

The material is extracted with cold water for a few days and precipitated with basic lead acetate ; the precipitate is treated with H_2S and after removing H_2S the lead treatment is repeated. The solution obtained is concentrated and the concentrated solution extracted with ether. The residue obtained by evaporating the ether is taken up with as little hot acetone as possible ; the solution is treated with an excess of anhydrous chloroform. The aconitic acid separates out on cooling and is recrystallised from absolute alcohol. M.P. 191° C.

Aconitic acid gives a red colour with acetic anhydride, which deepens rapidly and then becomes fuchsine red ; when heated it becomes bluish green and finally blue. If the fuchsine red solution is shaken with ether after the addition of water the ethereal layer becomes blue.

Detection and Separation of Vegetable Acids according to H. Franzen and his co-workers (*110*)

The extracts or juices are first precipitated with lead acetate solution and then with a solution of basic lead acetate. After

decanting off the liquid the precipitate is filtered quickly by suction and pressed as far as possible. The lead precipitates (chiefly to remove the sugars and inosite) are suspended as finely as possible in water, treated with CO_2 to saturation with vigorous shaking,[1] and filtered. The process is repeated and the filtrates combined with the previous one. The lead precipitates are then again suspended in water and decomposed by H_2S. The solution of the acids thus obtained is concentrated *in vacuo* to a thick syrup and the syrup treated with absolute alcohol in order to precipitate the pectins. The filtrate is evaporated to a thick syrup and again treated with absolute alcohol. If no more pectins separate out on repeating the process the alcoholic solution is treated with alcoholic hydrochloric acid (saturated in the cold) until the liquid contains 2·5% HCl. It is then heated to boiling for five hours. The solution of ester is evaporated *in vacuo* to a syrup and the residue shaken with ether. The ethereal solution of the ester is washed with a solution of potassium carbonate and dried over anhydrous sodium sulphate. The ether is distilled off and the residue submitted to fractional distillation *in vacuo*.

Boiling Points of the relevant Ethyl Esters

Diethyl oxalate - -	74° (11 mm.) ; 78° (15 mm.) ;
Diethyl succinate -	96° (10 mm.) ; 104°–105° (15 mm.);
Diethyl malate - -	129·2°–129·6° (12 mm.) ;
Diethyl tartrate - -	148° (9 mm.) ; 150° (11 mm.) ;
Triethyl citrate - -	169°–170° (10 mm.).

The esters are converted into the *hydrazides* and then into the *benzylidene compounds* in the following manner :

" A known amount of the ester is dissolved in thrice its volume of alcohol and a slight excess over the calculated equivalent amount of hydrazine-hydrate added. The mixture is kept for twenty-four hours at room temperature and the precipitated crystals are filtered by suction. The filtrate is then

[1] On a shaking machine suitable for the purpose, see H. Franzen, *Zeitschr. f. physiol. Chem.* 122, 86, 1922.

heated to boiling for two hours, kept at room temperature for twelve hours, and the crystals that may be precipitated filtered by suction. The filtrate is evaporated on the water-bath to dryness, the residue taken up with water, the solution acidified with a few drops of dilute HCl and benzaldehyde added in small amounts with vigorous shaking until its smell persists. The benzylidene compound is filtered by suction, dried and treated with ether to remove benzaldazine and purified further." The benzylidine compounds are also prepared from the aqueous solutions of the hydrazides in the same way.

The dihydrazide of oxalic acid is characterised by the fact that it is precipitated rapidly (1–2 minutes) even from dilute solutions.

	Melting Points	
	Of Hydrazides	Of Benzylidine Compounds
Oxalic acid dihydrazide - (Fine leaflets)	243°–244°	—
Succinic acid dihydrazide - (Long, flat needles)	167°–168°	233°–234°
Malic acid dihydrazide - - (Warts)	177°–178°	164°
Tartaric acid dihydrazide - (Needles)	183°	—
Citric acid trihydrazide - -	106°–107°	227°

Separations : (1) *Oxalic acid dihydrazide from succinic acid dihydrazide.* The mixture of dihydrazides is boiled with sufficient alcohol [1] to dissolve the succinic acid dihydrazide and filtered hot ; the oxalic acid dihydrazide remains undissolved.

(2) *Separation of malic acid dihydrazide from succinic acid dihydrazide.* The mixture of dihydrazides is boiled with sufficient alcohol [1] to dissolve the succinic acid dihydrazide ;

[1] One gm. of succinic acid dihydrazide dissolves in 63 ccs. of boiling alcohol.

the undissolved substance is the malic acid derivative.[1] Alternatively, the benzylidene compounds may be boiled with alcohol ; the malic acid derivative goes into solution.

(3) *Citric acid trihydrazide from malic acid dihydrazide.* The mixture is boiled with alcohol in which the citric acid trihydrazide dissolves.

The filtrate from the lead precipitate is treated with H_2S together with the liquids obtained by treatment with CO_2. The filtrate is concentrated, acidified with H_2SO_4 and extracted with ether. Succinic acid may separate out of the mass which remains behind on evaporating the ether. It is filtered by suction and the filtrate diluted with water. Any substances causing turbidity are shaken out with benzene, the aqueous solution is evaporated to a syrup and esterified as described above. In the first fractions one finds lactic acid ester (B.P. 49°–51° at 10 mm. pressure) ; the rest of the fractions may contain the same acids as those obtained by working out the lead precipitates. The benzylidine lactic acid hydrazide (fine colourless needles, M.P. 158°–159°) may be separated from the succinic acid derivative by hot methyl alcohol in which the lactic acid derivative is very easily soluble, while the succinic acid derivative is only soluble with difficulty. The hydrazide may also be separated by alcohol, in which the lactic acid hydrazide is very easily soluble.

Microchemical Tests for free and combined Vegetable Acids according to G. Klein and O. Werner (*106*).

0·5–5 mg. of the vegetable tissue is treated in a small copper capsule with 0·2–2 mg. of concentrated phosphoric acid, macerated with a needle, dried at 60°–70° C. for 15 minutes, and then submitted to fractional sublimation *in vacuo* for which a special apparatus is used.

[1] One gm. of malic acid dihydrazide dissolves in about 800 ccs. of boiling alcohol.

1. Fraction at 110°. Oxalic acid. The sublimate consists as a rule of long prisms at first which soon change to smaller crystals. It is identified by the strontium salt, or, in the case of small quantities, by the calcium salt.

2. Fraction at 130°. Succinic acid. The sublimate consists of small crystals which can be best identified by the lead salt.

3. Fraction at 145°. Anhydride of malic acid. At first an amorphous sublimate is obtained which on standing in the air forms efflorescent needles of malic acid after some time. It is identified by the silver salt. The sublimate is treated with a drop of 1–5% solution of $AgNO_3$, and placed in a warm oven at 40° C. in which there is a flat basin containing a 5% solution of ammonia. After 10–20 minutes small globules, discs, and, finally, rosettes of four-sided or octagonal crystals or sometimes also small needles are formed. The crystals of silver nitrate formed may be dissolved by breathing upon them.

4. Fraction at 170°. Anhydride of citric acid. This consists of an amorphous coating which can be converted into the silver salt as described for malic acid ; spherites of fine needles.

5. Fraction at 195°. Anhydride of tartaric acid. This consists of an amorphous sublimate from which tartaric acid gradually crystallises out on standing in the air in the form of wedge-shaped or powdery crystals. Ammonium tartrate (wedge-shaped) is prepared from these by keeping in an atmosphere of ammonia at 40° (see above), and from this the silver tartrate is obtained by means of silver nitrate and acetic acid. It crystallises in knee-shaped twin-crystals.

The quantitative estimation of organic acids may be carried out by Fleischer's process (see p. 120). The quantity of the acid-potassium tartrate which separates out can be estimated by titration with normal alkali. The calcium citrate is converted into the lead salt by dissolving in acetic acid and precipitating with lead acetate, the lead salt is washed with 50%

alcohol, suspended in water and decomposed by H_2S. After removing H_2S the free citric acid is titrated.

To estimate *oxalic acid* Fleischer treats the portion of the lead precipitate, insoluble in ammonia, with KOH, then adds ammonium sulphide, and, after acidification with acetic acid, boils and filters. The filtrate is mixed with H_2SO_4 and titrated with $KMnO_4$. A. Hilger (*103*) has worked out a method of estimation depending upon the (already mentioned) reduction of palladium chloride by malic acid. One gm. of malic acid reduces 0·294 gm. of palladium in the form of palladium chloride.

To estimate *succinic acid* the process of v. d. Heide and Steiner (see p. 122) is used, and the aqueous liquid obtained last is extracted in a perforator with ether for twelve hours. The contents of the flask are transferred to a beaker with about 20 ccs. of water and the ether evaporated off by allowing it to stand in a warm place. The liquid is next neutralised in the presence of phenolphthalein with $N/10$ caustic alkali (completely free from halogens), transferred to a 100 c.c. measuring flask, treated with an excess of $N/10$ $AgNO_3$ and filled up to the mark with thorough shaking. It is then filtered and 50 ccs. of the filtrate are titrated with $N/10$ ammonium thiocyanate in the usual way. One cc. of $N/10$ $AgNO_3 = 0{\cdot}0059$ gm. succinic acid.

On the estimation of tartaric acid in the presence of other organic acids see E. Bernhauer, *Oesterr. Chem. Ztg.* 31, 4; *Chem. Centralbl.* I, 1211, 1928.

On the estimation of citric acid see G. Jörgensen, *Zeitschr. Unterschg. d. Nahrgs.- u. Genussm.* 13, 241, 1907.

XIII

CARBOHYDRATES AND RELATED SUBSTANCES[1]

The large group of substances to be treated in this chapter may be divided into several sections according to their be-

[1] For general reactions of carbohydrates see p. 24.

haviour with solvents. Only those substances, however, will be dealt with whose distribution in the vegetable kingdom is fairly general.

1. *Easily soluble in cold water, soluble in hot alcohol*: glucose, fructose, cane sugar, maltose, mannite, inosite (galactose, mannose, arabinose, xylose).

II. *Easily soluble in cold water, insoluble in alcohol*: gums and similar substances.

III. *Soluble with difficulty in cold water, more easily soluble in hot, insoluble in alcohol*: vegetable mucins, pectins, lichenin, bassorin, glycogen, inulin, starch, xylan, amyloid.

IV. *Insoluble in cold and hot water, soluble in dilute alkalies*: difficultly soluble modifications of gums, galactoarabans, mannans, pentosans, hemicelluloses.

V. *Insoluble in the above mentioned solvents*: cellulose, lignin.[1]

I. *Inosite and mannite* are distinguished from the true sugars, glucose, fructose, cane sugar and maltose, by being less soluble in water. Inosite, which occurs less frequently in the vegetable kingdom, may be separated from the others by basic lead acetate with which it gives a precipitate. Mannite can be separated from a mixture with the above mentioned sugars by crystallising it out of hot water or alcohol.

These carbohydrates can be extracted from the plant material, treated previously with petroleum ether or ether, by cold water or hot dilute (about 50%) alcohol, the liquid being kept neutral with $CaCO_3$ to avoid hydrolysis. The concentrated liquid (freed from alcohol by evaporation) is purified by precipitation with lead acetate. The lead is removed by H_2S [2] and the acetic acid set free is neutralised in case a disaccharide is present. The liquid is evaporated to dryness, and the residue extracted with boiling ethyl or methyl alcohol. The sugars are precipitated on cooling the alcohol.

[1] A small portion of the so-called lignin may be soluble in NaOH.

[2] The excess of H_2S is removed by passing a current of air.

Further amounts of sugar are obtained by evaporating the solution and also by precipitation with ether. It should be noted here that laevulose is considerably more soluble in alcohol and ether-alcohol than the other sugars concerned, and more of it will therefore remain in the mother-liquor than of the others. The sugar so obtained is purified further by repeating the operations and decolorising with animal charcoal. A portion of the syrup or the precipitate which is nearly always obtained in the amorphous state is kept for crystallisation *in vacuo* over H_2SO_4. The spontaneous crystallisation of sugars is often very slow.[1] Cane sugar, dextrose and maltose crystallise more readily than laevulose.

An indication as to the sugars that may be present *in syrups* is given by the process of Widtsoe and Tollens (*111*) : A few crystals of the sugar expected are put into a little of the syrup on a slide, and it is observed (preferably under the microscope) whether the crystals increase or disappear. In the former case the sugar present is the same as that added to the syrup. Further information concerning the composition of the syrup is obtained from its behaviour with reagents.

The method of detecting non-reducing sugars in the presence of reducing sugars has already been discussed (see p. 20). Fehling's solution (and similar alkaline copper solutions) are reduced by glucose, fructose and maltose, but they are not reduced by cane sugar, at least by boiling once. On boiling with Fehling's solution maltose is partially converted into substances which after boiling with acids are again capable of reducing alkaline copper solutions. Maltose in the presence of other sugars can be detected by its behaviour with copper acetate solution (*Barfoed's Reagent*)[2] followed by a neutral solution of basic copper carbonate in Seignette's salt. Barfoed's Reagent is reduced by boiling with glucose and fructose. The

[1] For the promotion of crystallisation by inoculation see p. 14.

[2] A solution of 13·3 gms. of copper acetate in 200 ccs. of 1% acetic acid or a solution of 50 gms. of copper acetate, 50 gms. of sodium acetate and 5 ccs. of glacial acetic acid in 1 litre.

maltose, which remains intact, is then allowed to act in neutral solution with the other reagent. Maltose reduces this reagent on boiling while cane sugar remains unchanged.

If a red colour is produced by slightly warming the sugar on the water-bath with resorcin and HCl it contains fructose or a substance combined with fructose. The reaction is only conclusive when a control experiment carried out in the same way with glucose is negative.

The identification of fructose in the presence of glucose is done most simply by *Pieraert's Reagent*,[1] which is reduced only by fructose on allowing to act for twelve hours.

Formation of saccharic acid on oxidation of the sugar with HNO_3 shows the presence of glucose or a substance combined with glucose.[2]

The oxidation is carried out by evaporating the sugar with HNO_3 (sp. gr. 1·15) on the water bath (*112*). The aqueous solution of the residue is exactly neutralised while hot with K_2CO_3, and the acid-potassium saccharate formed from the glucose is precipitated by evaporation and addition of acetic acid. It is purified by recrystallisation. For further identification of saccharic acid the Ag salt is prepared by precipitating a solution of the acid-potassium saccharate neutralised with ammonia with a solution of silver nitrate.

On the preparation of crystalline saccharic acid see K. Rehorst, *Ber. deutsch. chem. Gesellsch.* **61**, 163, 1928.

With mixtures of several sugars an attempt should be made either to separate the sugars from each other or to prepare their characteristic derivatives. As the sugars can be prepared back from some of their derivatives a separation of sugars can be effected in a few cases by this means.

[1] Pure copper hydroxide is gradually added to a solution of 12 gms. of glycocoll in hot water and warmed for five minutes on the water bath until it is dissolved ; after cooling to 60°, 50 gms. of powdered potassium carbonate are added and the solution made up to 1 litre with water and filtered.

[2] At least in the absence of glucuronic acid which is likewise oxidised to saccharic acid.

A separation of cane sugar from other sugars[1] can be made with a solution of strontium hydrate. The alcoholic solution of the mixture of sugars is boiled for half an hour with a hot saturated aqueous solution of strontium hydrate, the precipitate washed with alcohol and pressed between filter-papers. It is then boiled for half an hour with hot strontium solution and filtered through a hot filter. The residue is pressed between filter papers to remove the mother-liquor, suspended in water and decomposed by carbon dioxide. The liquid is evaporated off, taken up with hot alcohol and allowed to crystallise by evaporation.

To prepare cane sugar from plants E. Winterstein used the following method (*112a*). After previous extraction with ether the material is boiled with ten times its bulk of 95% alcohol with the addition of calcium carbonate. The filtered extract is concentrated, taken up with water and stirred for six days with a slight excess of freshly prepared lead hydroxide, adding some solution of aluminium sulphate from time to time. The liquid is filtered by suction, the filtrate treated with H_2S and mixed with the same volume of alcohol. The filtrate from PbS is evaporated *in vacuo* and the residue extracted three times with hot methyl alcohol. The clear solution is decanted from the precipitate obtained on cooling, and treated with benzene, acetone or toluene until highly turbid. The liquid is decanted off and allowed to stand, whereupon cane sugar crystallises out.

From a mixture of glucose, fructose and cane sugar, the cane sugar can be isolated by decomposing the other two sugars by boiling with excess of $Ca(OH)_2$. The liquid is then well cooled and filtered, decomposed with carbon dioxide, purified with animal charcoal, evaporated, and so on.

[1]According to Congdon and Joung (*113*) cane sugar can be freed from glucose by treating with acetic ether, which can also be used for the separation of other sugars as shown by the following data : 100 parts of acetic ether dissolve 4 parts of maltose, 5 parts of lactose, 6 parts of cane sugar, 14 parts of galactose, 18 parts of mannose, 32 parts of glucose (anhydrous), 37 parts of arabinose and 42 parts of fructose.

To identify cane sugar in the presence of reducing sugars the sugar solution (which should not contain more than 2% of reducing sugars) may be heated, according to Jolles (*114*), with caustic soda (total concentration N/10) either for twenty-four hours in a thermostat at 37° or for three-quarters of an hour under a reflux condenser or in a Lintner pressure bottle, both the latter over a boiling-water bath. The reducing sugars lose all their optical activity or all but a trace; the cane sugar remains unchanged.

According to Rothenfuszer (*115*), the *monosaccharides* may also be *decomposed by hydrogen peroxide* in alkaline solution and the *cane sugar identified by means of diphenylamine*. For example, 10 ccs. of a solution containing 7% of the total sugar are treated with 15 ccs. of 20% KOH, made up to 50 ccs. with water and then treated with 15 ccs. of 3% hydrogen peroxide. The mixture is heated in a thin glass dish or in a spouted nickel capsule over a boiling-water bath. If there is a yellow colour after five minutes hydrogen peroxide is added drop by drop until decolorised. After about 10–12 minutes some infusorial earth is added with constant stirring. After a total interval of twenty minutes the mixture is filtered, 5 ccs. of the colourless filtrate are treated with *Diphenylamine Reagent*,[1] and the test-tube is kept for eight to nine minutes over a boiling-water bath. A blue colour shows the presence of cane sugar.

For the identification and estimation of cane sugar see also p. 64; also, H. Riffart and C. Pyriki, *Zeitschr. f. Untersuchg. der Nahrungs- und Genussm.* 48, 197, 1924.

Glucose and fructose can be separated by their behaviour with caustic lime, with which fructose forms a compound soluble only with difficulty in water, while glucose forms a water-soluble compound. To a roughly 10% solution of the sugar $Ca(OH)_2$ is added in the cold in the proportion of six parts of hydroxide to ten parts of sugar. The mixture is shaken

[1] 20 ccs. of a 10% alcoholic solution of diphenylamine, 60 ccs. of glacial acetic acid and 120 ccs. of concentrated HCl.

as long as the lime-fructose compound is precipitated. After filtration both the filtrate and the precipitate are decomposed by carbon dioxide or oxalic acid, and on evaporation of the resulting solutions glucose is obtained from the former and fructose from the latter.

Derivatives which are specially characteristic of the sugars containing aldehyde or ketone groups (glucose, fructose, maltose) are formed by the action of phenylhydrazine and compounds of similar composition. With phenylhydrazine itself the sugars give phenylhydrazones in the cold and phenylosazones by heat, the latter forming yellow crystals soluble in water only with more or less difficulty.

The phenylosazones are formed when the sugar solution is heated on the water bath with phenylhydrazine hydrochloride and sodium acetate. Thus phenylglucosazone (*116*) is obtained when one part of glucose, two parts of phenylhydrazine hydrochloride, three parts of sodium acetate and twenty parts of water are treated in the above way (E. Fischer). It is purified by recrystallisation from strong or dilute alcohol. While the osazones of glucose and fructose are precipitated even from the hot aqueous liquid, maltosazone (when not too concentrated) may remain in solution while warm and first separate out on cooling. Maltosazone can be separated from the osazones of the other two sugars by taking advantage of this behaviour. The melting point of maltosazone is 206°; glucose and fructose give the same osazone melting at 204°–205°.

On the identification of *glucose and rhamnose* in the presence of each other see p. 65.

Glucose can be identified in the presence of fructose by converting it into saccharic acid and its silver salt. Another method is to decompose the fructose by heating 50 ccs. of sugar solution with 10 ccs. of 5*N* HCl for about seven hours. The glucose remains almost unchanged.

To test for glucose in the presence of fructose the hydrazone (*117*) (M.P. 162°) obtained by the action of diphenylhydrazine upon glucose can also be used, as also the glucose benzhydrazide (*118*), melting at 171°–172°, obtained by heating both the components in the presence of alcohol for five to six hours under a reflux condenser. The compound can be hydrolysed back into glucose and benzhydrazide by means of water. The latter can be removed by the addition of benzaldehyde, with which it forms an insoluble compound.

For the identification of glucose it can also be converted into *β-methyl glucoside*. For example, 0·9 gm. of the sugar is dissolved in 100 ccs. of 70% methyl alcohol, a concentrated solution of 1 gm. of emulsin is added and the solution tested polarimetrically from time to time. When the rotation does not change any more, and equilibrium is therefore reached, it is filtered, evaporated *in vacuo*, the residue is boiled with a mixture of 10 ccs. of 95% alcohol and 25 ccs. acetic ether. The β-methyl glucoside separates out on cooling and is identified by the melting point (109°–111°) and rotation : $[\alpha]_D^{20} = -32{\cdot}25°$ (1% aqueous solution).

It is not, however, essential to start with pure sugar for the preparation of the β-methyl glucoside, and one can also use plant extracts sufficiently purified. It is dissolved in so much 70% methyl alcohol as to give a 1% solution of the sugar and the solution treated as described before (*119*).

To identify fructose, the osazones, which are given by asymmetric secondary hydrazines (*120*), may be used, since only the ketoses but not the aldoses form osazones with these substances. (Melting point of fructose-α-methyl phenylosazone, 161°–162°.)

Of the hydrazones one may mention the benzyl phenylhydrazones (*121*), well crystallised compounds which are obtained by adding an absolute alcoholic solution of benzyl phenylhydrazine to a concentrated solution of a sugar capable of yielding

a hydrazone. *The original sugar can be recovered from the hydrazone* by means of formaldehyde or benzaldehyde. In using formaldehyde, 1 gm. of the hydrazone is dissolved in 2–3 ccs. of 30–40% hot formaldehyde solution and warmed on the water bath. The solution becomes turbid, and the formaldehyde hydrazone separates out as a heavy oil, which is removed by extraction with ether. Excess of formaldehyde is removed by evaporation, and some metaformaldehyde which may be present is removed by dissolving the syrup in absolute alcohol.

The decomposition of the hydrazone by benzaldehyde is done in the same way.

If the sugars have been isolated in sufficiently pure condition their most important *properties* are ascertained and compared with those of the known sugars. Of special importance are : crystalline form, behaviour with copper acetate and alkaline copper solutions, fermentability, effect on polarised light, behaviour on treatment with HCl, HNO_3, and hydrazines, especially phenylhydrazine, and the melting points of the derivatives formed. The phenylhydrazine reaction can be done in a quantitative way, since the sugars which react with phenylhydrazine yield definite amounts of osazones under definite conditions (*122*).

Of the *specific reactions* of the individual sugars the following may be mentioned :

Cane sugar: (*a*) If a porcelain plate is sprinkled with the sugar solution, the plate placed upon a boiling-water bath and a few drops of 1% solution of arsenic acid added, an initial red colour is formed, and later a brilliant purple (*Maumené's Reaction*). (*b*) Since it contains laevulose it gives a red colour when warmed with HCl and resorcin. (*c*) The rose colour formed when 0·1 gm. cane sugar dissolved in 10 ccs. of fuming HCl is shaken with 20 ccs. of sesame oil also depends upon the presence of the laevulose group. (*d*) It is hydrolysed by saccharase into dextrose and laevulose. (*e*) It is dextro-rotatory.

Glucose (dextrose, grape sugar): (*a*) It gives saccharic acid on oxidation with nitric acid. (*b*) It is dextro-rotatory. (*c*) The lime-compound is soluble in water. (*d*) It does not give a red colour when carefully warmed for a short time with resorcin or phloroglucin and HCl. (*e*) It reduces alkaline copper, mercury and bismuth solutions.

Fructose (laevulose): (*a*) It gives no saccharic acid on oxidation with HNO_3. (*b*) It is laevo-rotatory. (*c*) The lime-compound is soluble with difficulty in water. (*d*) It gives a rose-red colour on warming with resorcin and HCl, a yellowish-brown colour with phloroglucin and HCl. (*e*) It shows the same reduction reactions as glucose. (*f*) It gives the reactions mentioned in (*a*) and (*c*) under cane sugar.

Cane sugar, glucose and fructose are fermented by yeast.

Maltose: (*a*) With brewer's yeast and in its reduction reactions it behaves like glucose with the exception that it is not reduced by *Barfoed's Reagent* (see p. 134). (*b*) Unlike cane sugar, dextrose and laevulose it is not fermented by Saccharomyces Marxianus. (*c*) The phenylosazone melts at 206° and is considerably more soluble in warm water than the glucosazone.

Mannite: (*a*) The very weak laevo-rotation is changed by alkaline arsenite solution into a strong dextro-rotation. (*b*) It is oxidised by hydrogen peroxide in the presence of ferrous sulphate to mannose, which is characterised by its phenylhydrazone (M.P. 199°), which is soluble with difficulty. (*c*) It is not reducing. (*d*) If mannite is dissolved in fuming HCl and shaken with benzaldehyde, tribenzylidine mannite separates out gradually. M.P. 207° (218°–222°).

Inosite: (*a*) Optically inactive, does not reduce alkaline copper solution and is more resistant to acids and alkalies than the other substances of this group. (*b*) If a drop of a solution of mercuric nitrate is added to a concentrated solution of inosite a precipitate is obtained which becomes dark rose-red on warming and disappears again on cooling (*Reaction of Gallois*).

(*c*) If a little inosite is evaporated with nitric acid and ammonia or sodium acetate and then barium chloride added the mass assumes on standing a reddish coloration (*Reaction of Scherer-Seidel*).

The quantitative estimation of sugars is based upon their behaviour with polarised light, with alkaline copper solution and with yeast. Non-reducing sugars like cane sugar are inverted before they are estimated with alkaline copper solution. By suitable combination of the methods two (or even more) sugars can be estimated in the presence of each other.

Thus, for example, the sum of zymohexoses and galactose is estimated at $p_H = 5{\cdot}5$ (phosphate buffer) by fermenting with "Löwenbräu" yeast for twenty-four hours, the yeast having been previously laid out in the air for twenty-four hours to eliminate autofermentation; zymosaccharoses alone are then estimated by means of Schizosaccharomyces Pombe at p_H 3·7 (acetate buffer). The difference gives galactose (*123*).

Glucose can be estimated in presence of fructose and cane sugar by oxidation with hypoiodite (Method of Willstätter and Schudel modified by Auerbach and Bodländer) (*124*).

On the estimation of galactose see pp. 143 and 147, of pentoses see p. 145, and of methyl pentoses see p. 146.

For further information on the estimation of sugars see, among others, the text books on pharmaceutical chemistry and chemistry of food-stuffs, A. W. van der Haar, *Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren* (Verlag Gebr. Bornträger, Berlin 1920), and E. O. v. Lippmann, *Die Chemie der Zuckerarten* (Verlag Vieweg & Sohn, Braunschweig), 3 Aufl. 1904.

II. Gums and similar substances. These substances are mostly compounds of carbohydrates of acid nature (arabinic acid) with calcium or magnesium. If the arabinic acid is precipitated from an aqueous solution of the gum with HCl and alcohol, it is changed on drying into an insoluble modification,

which often occurs in the vegetable kingdom either free or in the form of a salt, together with the soluble modification. The insoluble modification is changed again into the soluble modification by the action of weak alkalies (lime or baryta water).

The gum may be dissolved in cold water, the foreign constituents precipitated if necessary with lead acetate and the gum precipitated with basic lead acetate. The lead precipitates are decomposed by H_2S in the usual manner. It is often difficult to separate the solution of gum from the PbS. The lead method should, therefore, be avoided as far as possible and the gum precipitated with alcohol, since it is soluble only with great difficulty even in dilute alcohol. In order to purify the organic portion of the gum the precipitate is redissolved in a little water, to which a little HCl is added, precipitated again with alcohol, and the operations repeated until the precipitate is free from inorganic constituents. To attain this object dialysis or electro-osmosis (see p. 115) may also be used.

The gums can scarcely be distinguished from each other in their physical properties ; they are amorphous and do not give any crystalline derivatives, and they behave on the whole in the same manner with precipitating reagents. The investigation, therefore, turns chiefly upon ascertaining the substances formed by acting upon them with HCl or H_2SO_4 (hydrolysis) and by their oxidation with HNO_3. The methods of investigation are the same as those used for vegetable mucins, pectins and membrane substances proper which will be described later.

If a substance of this nature gives saccharic acid on oxidation according to the method given on p. 135 it must contain glucose ; if, on the other hand, mucic acid, which is soluble with difficulty in water, is formed, a galactose residue must be present in the molecule.[1] For the *preparation and estimation*

[1] Galacturonic acid also gives mucic acid on oxidation ; quercitol and dulcitol behave likewise.

of mucic acid (*125*) which may be formed Tollens gives the following directions: 5 gms. of sugar are warmed on the steam bath with 60 ccs. of HNO_3 of specific gravity 1·15 in a glass beaker of about 5·7 cm. diameter until the liquid, which has originally a depth of 2·5 cm., has evaporated down to 8–9 mm. The evaporated mass is stirred next morning with 10 ccs. of water, filtered off after twenty-four hours through a filter dried at 100°, washed with 25 ccs. of water, dried at 100° and weighed. The melting point of mucic acid is 211°–212°. Since galactose yields about 75% of mucic acid on oxidation one can calculate from the amount of mucic acid obtained how much of the substance under investigation consisted of galactose. Cf. also p. 148.

On boiling with HCl or H_2SO_4 the substances mentioned above are transformed first into sugars. On further action of acids the sugars behave differently: hexoses yield oxy-methyl furfural and laevulinic acid[1]; pentoses, furfural, and methyl pentoses, methyl-furfural.

To test for laevulinic acid (*126*) the substance to be investigated is heated for twenty hours on the water bath with about 20% HCl in a flask fitted with a rubber stopper carrying a long glass tube. When the action is over it is filtered, and the filtrate is shaken four times with an equal volume of ether. The residue, which remains on evaporating the ether, is heated in a steam drying oven for half to one hour. A small portion is tested with iodine and NaOH to determine whether iodoform is formed. If negative, laevulinic acid is absent; if positive, the residue is dissolved in water, digested with zinc oxide, decolorised with animal charcoal, and the crystals of zinc laevulinate obtained by evaporation are converted by precipitating its concentrated solution with silver nitrate into the silver salt which possesses a characteristic crystalline form.

[1] Laevulinic acid may also be formed from glucosamine, chitin and chitose.

Reactions of Pentoses (Arabinose, Xylose).

(*a*) The solution of the sugar is treated with so much HCl (sp. gr. 1·19 = 38%) that the content of HCl in the solution amounts to about 20%. If the solution is then heated with a little phloroglucin there is first a cherry-red colour and then a darker precipitate. If the precipitate is washed with a little water and dissolved in alcohol the solution shows on spectroscopic examination an absorption band between D and E (*127*).

If orcin is used instead of phloroglucin a bluish-green colour is produced and a similar precipitate. The absorption band lies between C and D, and is contiguous with the D line (*128*).

(*b*) If the sugar solution is distilled, after mixing with so much HCl that the resulting solution contains 12% HCl, and 5 ccs. of HCl are added every time 5 ccs. of liquid have distilled over, the distillate shows the following *reactions* (*for furfural*):

1. With a mixture of equal parts of aniline and glacial acetic acid a red colour is formed (*129*).

2. If the distillate is mixed with an equal volume of concentrated HCl and a few small crystals of resorcin the following phenomenon is observed under the spectroscope: while the liquid becomes gradually darker in colour, an absorption band is formed in the red which gradually increases in breadth until the clear middle space is filled up (*130*).

Tollens and his co-workers (*131*), as also Stone (*132*), have worked out methods for the quantitative estimation of furfural, and from it pentoses and pentosans, on the basis of the phenyl hydrazine precipitate.

On the phloroglucide method cf. *Journ. f. Landwirtschaft*, 48, 357, and *Zschr. f. angew. Chemie*, 15, 477-482, 1902.

On the estimation of pentoses and pentosans compare also N. C. Pervier and R. A. Gortner, *Ind. and Engin. Chem.* 15, 1167, 1255, 1923; *Chem. Zentralbl.* I, 2617, 2618, 1924, and F. W. Klingstedt, *Zeitschr. analyt. Chem.* 66, 129, 1925.

Reactions of Methyl Pentoses (Fucose, Rhamnose, Rhodeose).

(*a*) If a methyl-pentose is warmed with 10 ccs. of concentrated HCl and 1–2 ccs. of pure acetone on a boiling-water bath, a raspberry-red colour ensues. The liquid shows an absorption band in the yellow.[1]

(*b*) If methyl pentoses are distilled as described under pentoses the distillate shows the following *reactions* (*for methyl furfural*):

1. The distillate, warmed with half its volume of 38% HCl and 1–2 ccs. of acetone, shows the phenomena described in (*a*).

2. If the distillate is heated with an equal volume of 38% HCl, the solution examined spectroscopically shows an absorption band between the green and blue (*133*).

For the tests for rhamnose in the presence of glucose see p. 65.

Tollens and his students have worked out methods for the quantitative estimation of methyl pentoses even in the presence of pentoses (*134*).

Among the sugars, which may be formed by the hydrolysis of gums, vegetable mucins, membrane-substances, etc. (in addition to dextrose and laevulose), *mannose*, *galactose*, *arabinose* and *xylose* may also be mentioned.

The closely related *glucuronic acid* and *galacturonic acid* may also be formed besides the sugars.

Mannose (M.P. 132°–133°) is dextro-rotatory; it gives a precipitate with basic lead acetate in a solution which is not too dilute (distinction from other monosaccharides) and is fermented by yeast.

The phenylhydrazone (M.P. 199°) is characteristic. It is obtained by treating an aqueous solution of the sugar with E. Fischer's phenylhydrazine solution (see p. 138) or with a

[1] Pentoses give a similar colour, which, however, disappears after warming for ten minutes at the most, after which no absorption band can be observed.

solution of free phenylhydrazine in double the quantity of 25% acetic acid. It is crystallised first from water and then from 60% alcohol and washed with alcohol and ether.

Galactose (M.P. 118°–120°) is dextro-rotatory and is fermented at $p_H = 5{\cdot}5$ (phosphate buffer) by Munich " Löwenbräu " yeast. It can be identified as mucic acid, or as α-methyl-phenylhydrazone (M.P. about 190°) or diphenyl-methane-dimethyl-dihydrazone (M.P. 185°). To prepare the first, 0·4 gm. of as-methyl-phenylhydrazine is added to a solution of 0·5 gm. of galactose in 3 ccs. of water, and then alcohol added until the solution becomes clear. It is crystallised from hot water or 30% alcohol.

The *o*-tolyl-hydrazone (*135*) is also very suitable for the identification of galactose even in the presence of other sugars. To prepare this a solution of one part of galactose in an equal bulk of water is heated with one part of *o*-tolyl-hydrazine, dissolved in twenty parts of absolute alcohol, for half an hour on a boiling-water bath. The hydrazone is filtered by suction after twenty-four hours, washed with alcohol and ether and recrystallised from 95% alcohol. M.P. 176°.

Galactose can also be identified by converting it into β-ethyl-galactoside, especially in the presence of arabinose. M. Bridel and J. Charpentier (*136*) identified it among the products of hydrolysis of gum arabic in the following way : the liquid obtained by hydrolysis with sulphuric acid was treated with $CaCO_3$, filtered from the calcium sulphate and concentrated *in vacuo* to 150 ccs. It was filtered again and evaporated *in vacuo* to dryness. The extract was boiled with 500 ccs. of acetic ether in order to remove impurities, dried again warm *in vacuo*, the residue dissolved in so much 70% alcohol that a 5% solution of the extract is obtained. Five gms. of emulsin were added to 500 ccs. of the liquid, and the addition of emulsin repeated on the 10th, 20th, 41st and 75th days, keeping the solution at 37° C.

After eighty-nine days the liquid was worked out. It was

filtered from emulsin and evaporated to dryness *in vacuo*. The residue was dissolved in water and submitted to the hydrocyanic acid process (see p. 56) in order to remove the arabinose. The liquid freed from PbS was evaporated *in vacuo* to dryness and the residue boiled repeatedly with acetic ether. On cooling the β-ethyl-galactoside crystallised out. It was filtered, washed with absolute alcohol and dried in the air. M.P. (Maquenne's block) 159°–160°, $[\alpha]_D = -4°{\cdot}40$ (in 2·27% solution).

On the quantitative estimation of galactose by the mucic acid process see A. W. van der Haar, *Biochem. Zeitschr.* 81, 263, 1917, and *Anleitung zum Nachweis der Monosaccharide usw.* (see p. 142), p. 123.

d-Xylose (M.P. 148°, 154°) is dextro-rotatory and is not fermented by yeast. It gives a purple-red colour with an ethereal solution of HBr after one to two hours.

A characteristic compound of xylose is the *cadmium-bromo-xylonate* (*137*), which is insoluble in alcohol and is formed by mixing 5 gms. of the syrup to be investigated with 15 gms. of water, 6 gms. of cadmium carbonate and 3 gms. of bromine, heating after twenty hours and filtering while boiling. From the concentrated filtrate the compound is precipitated by alcohol as boat-shaped crystals.

The xylose-*p*-nitro-phenyl-hydrazone (M.P. 190°–191°) is also very characteristic.

l-Arabinose (M.P. 160°) is dextro-rotatory and is not fermented by yeast. The α-benzyl-phenylhydrazone (M.P. 174°) and the *p*-bromo-phenylhydrazone (M.P. 161°–163°) are characteristic. They are prepared by treating a 1% solution of the pentose with so much of a solution, consisting of one part of the hydrazine, 3·5 parts of 50% acetic acid and twelve parts of water, that two parts of the hydrazine are used for one part of arabinose.

A separation of xylose and arabinose can be effected by β-naphthyl-hydrazine (*138*). If the method described above is followed the arabinose-β-naphthyl-hydrazone (M.P. 176°–177°

corr.) crystallises out, while the analogous compound of xylose (M.P. 124°) can be obtained from the mother-liquor.

d-Glucuronic acid, $CHO(CHOH)_4 . COOH$. In pure condition it forms needles, M.P. 154°. It reduces Fehling's solution. It is not fermented by yeast. It is easily transformed into the anhydride, glucuron, M.P. 175°–178°. It is precipitated by basic lead acetate but not by lead acetate ; it is also precipitated by baryta water in concentrated solution. Nitric acid or bromine water oxidises it to saccharic acid. On warming with HCl it gives furfural.

The difficultly soluble cinchonine salt and *p*-bromo-phenyl-osazone-barium-glucuronate are also suitable for its identification.

The acid is neutralised with baryta water, the solution is treated with the necessary quantity of pure *p*-bromo-phenyl-hydrazine-hydrochloride and barium acetate (4 gms. of the hydrochloride and 0·6 gm. of barium acetate for 1 gm. of glucuronic acid), warmed for a short time on the water bath and rapidly filtered off. The filtrate is then mixed with a little acetic ether and heated on the water bath for half an hour. The crystals formed ($C_{18}H_{17}O_5N_4Br_2Ba$ = *p*-bromo-phenyl-osazone-barium-glucuronate, needles, M.P. 216°) are washed with water, alcohol and ether.

To test for glucuronic acid in the presence of sugars, one may proceed according to v. d. Haar (*139*) as follows : The solution is evaporated with $BaCO_3$ to dryness, the mass is extracted repeatedly by boiling with alcohol and the insoluble residue is then extracted with water, which then dissolves the barium salt of glucuronic acid. The glucuronic acid is set free by careful addition of dilute H_2SO_4. *The naphthoresorcin reaction* according to Tollens is carried out with this solution : a mixture of the solution with an equal amount of 38% HCl is boiled, and after adding 0·1 gm. of naphthoresorcin it is boiled again for half a minute. After cooling to 50° it is shaken with benzene. A violet solution is obtained with an absorption band at D.

The quantitative estimation of glucuronic acid can be done either by the phloroglucide method (see p. 145 ; according to Lefèvre one part of furfural phloroglucide = three parts of glucuronic acid lactone) or by the estimation of the carbon dioxide obtained by distillation with HCl (Lefèvre's process). For details of the latter process see K. U. Lefèvre, *Untersuchungen über die Glykuronsäure* (Diss. Göttingen, 1907), and A. W. van der Haar (*loc. cit.* see p. 142), p. 71.

Galacturonic acid. Behaves like glucuronic acid in most of its properties (including the naphthoresorcin reaction) but is distinguished from it by the fact that its oxidation yields mucic acid. It differs from galactose in that it is oxidised by bromine water.

III. Closely related to the gums are a few *substances with* gummy and *mucin-like nature*. To these belong the *vegetable mucins* which are more or less soluble in water. Of the mucins, a few, like *bassorin* and *pectins*, only swell up in water. The preparation of these substances, partly precipitated by lead acetate, is generally accomplished by extraction with hot water and precipitation with alcohol. A few mucins can be salted out of their aqueous solutions. They are freed from ash by the methods given for gums.

The **pectins** are altered even by hot water. For further details on these and their investigation see F. Ehrlich, *Zeitschr. f. angew. Chemie*, 40, 1305, 1927.

To prepare **amyloids**, which have been obtained from the seeds of Leguminosae and Tropaeolum majus, the seeds are first extracted with ether, hot alcohol, dilute ammonia and cold 1% NaOH, the residue is washed with water and the amyloid then dissolved by boiling water, from which it is precipitated by alcohol. Amyloid is coloured blue with iodine.

Glycogen, which occurs in many fungi, can be precipitated from its opalescent solutions (which clear on addition of acetic acid or KOH) by means of alcohol. It is coloured red to brown with iodine, and yields dextrose on hydrolysis.

To obtain **inulin**, which occurs in the underground organs of many Compositae, the material is extracted with hot water with the addition of calcium carbonate. The extract is allowed to settle to become clear, filtered, and the filtrate evaporated until inulin begins to separate out. The mucin, etc., may also be first precipitated with basic lead acetate, the Pb removed by H_2S, the H_2S removed by a current of CO_2, the solution neutralised with calcium carbonate and the inulin again precipitated by evaporation. It is further purified by repeatedly taking up with hot water and precipitating by cooling and then washing with alcohol and ether. The purification of inulin can be also done by precipitation with baryta water.

On the identification of inulin by a mycological process see A. Castellani, and F. E. Taylor, *Biochem. Journal*, **16**, 655; *Chem. Zentralbl.* II, 381, 1923.

Inulin. Laevo-rotatory. The aqueous solution turns yellow with iodine. It does not reduce alkaline copper solution. The aqueous solution gives precipitates with the hydroxides of the alkaline earths. Hydrolysis with dilute acids gives only fructose.

On the estimation of inulin see *Zeitschr. f. Untersuchg. d. Nahrungs- und Genussm.* **5**, 81, 1902.

Lichenin (lichen cellulose). It is allied to cellulose. Gives a colloidal solution with water. Optically inactive. Soluble in dilute alkalies. Solid lichenin turns blue-black with iodine; the aqueous solution is not coloured by iodine. Fehling's solution is reduced only in traces. It is hydrolysed by lichenase to cello-biose, and if cello-biase (an enzyme from the snail, Helix pomatia) is also present, to glucose.

The presence of **starch** can be best ascertained by the microscope, by which the different forms can be easily seen, and also by the characteristic blue colour with iodine.[1] It is insoluble in cold water, is converted into a paste with hot water and is present in this state in the hot aqueous solution. Starch

[1] A few varieties of starch give a brownish-red colour with iodine.

goes into solution more easily, if not unchanged, when the crude material is treated with acids or alkalies. For the preparation of starch, which is only easily possible from materials which are rich in it, the well-known *mechanical method* is used. From the powdered material the starch is washed out with water, allowed to settle, washed several times with water and dried at a low temperature. To estimate starch it is generally converted by hydrolysis into dextrose, and the amount of the latter determined polarimetrically or by alkaline copper solution. None of these methods is, however, very exact, chiefly because the simultaneous hydrolysis of other substances yielding reducing sugars cannot be avoided.

As regards other methods of estimation I may refer to the colorimetric method of Dennstedt and Voigtländer (*140*), the gravimetric method of Baumert and Bode (*141*), and the polarimetric method of C. Mannich and K. Lenz (*142*).

Xylan, or wood-gum, though not so widely distributed as starch is, is found in many plants. It gives an opalescent solution with cold water and a clear solution with hot water. It is prepared by extracting the crude material first with 1–2% ammonia, then dissolving the xylan in caustic soda and precipitating with alcohol. It is purified by washing with alcohol containing HCl and with alcohol and ether. It gives a precipitate with basic lead acetate, and hydrolysis yields xylose.

IV. To this class belong the *insoluble modifications of gums*, which are dissolved by treatment with alkalies and then mostly behave like the soluble modifications.

A few membrane-substances, like mannan, pentosane and hemi-celluloses, must, however, here be referred to further. It may also be mentioned that there is no absolute significance in the differentiation of this group and the two following by their solubility in weak alkalies, since the members of the next groups are also slightly soluble in weak alkalies. The behaviour of these substances with alkalies is complicated by the fact that

they may contain incrusting substances which are extracted by the action of solvents.

Mannan is found in many seeds, and gives mannose together with other sugars on hydrolysis. Mannose is characterised by the hydrazone (see p. 146), which is soluble with difficulty in the cold. The substances designated as mannans in the literature do not all appear to be identical.[1]

The "Hemi-celluloses" are soluble in 5% NaOH,[2] and are precipitated from these solutions by the addition of HCl and alcohol like the other members of this and neighbouring groups. They are easily hydrolysed by dilute HCl and are transformed thereby into sugars, among which may be found galactose, xylose, and others in addition to glucose.

The Pentosans are similar to the hemi-celluloses in their general behaviour. On hydrolysis they yield at first pentoses (or methyl pentoses) and then larger amounts of furfural and methyl furfural, which are used for identification and quantitative estimation (see p. 145).

On the preparation of **galacto araban**, soluble in hot 2% solution of KOH, see A. Heiduschka, and H. Tettenborn, *Biochem. Ztschr.* **189**, 202, 1927.

V. The fifth group contains the **Oxy-celluloses**,[3] which are formed by the oxidation of cellulose, and occur in plants. They react with salts of phenylhydrazine in the cold with the formation of a yellow colour, which becomes more intense on heating to 70°. They are coloured red by fuchsine-sulphur dioxide and reduce boiling alkaline copper solution. The oxy-celluloses are dissolved by sulphuric acid of specific gravity 1·5 saturated with HCl gas, prepared by mixing 52 ccs. of

[1] On mannans see M. Lüdke, *Annal. Chemie*, 456, 201, 1927.

[2] Many hemi-celluloses, *e.g.* those of wheat, are, according to their method of isolation, soluble in hot water, and separate out on cooling in the amorphous state. The term "hemi-cellulose" must be defined for every raw substance (E. Heuser).

[3] According to K. Hess (*Annal. Chem.* 455, 214, 1927), the chemistry of oxy-cellulose should be differentiated from the chemistry of cellulose, but Nastukov believes them to be the same (*Ber. deutsch. chem. Gesellschaft*, 60, 2591, 1927).

concentrated sulphuric acid, 23 ccs. of HCl and 25 ccs. of water.

VI. The substances which remain behind after treatment of the *membrane-matter* with 5% NaOH are the **celluloses** and part of the so-called **lignin** (also belonging to the pentosans).

The separation of lignin from cellulose is effected, according to Hoffmeister (*143*), by ammoniacal copper oxide, or *Schweizer's Reagent*,[1] which dissolves the celluloses. By evaporating the solution to dryness, removing the copper by water containing HCl and HNO_3, washing the residue with ammonia, alcohol and NaOH, the cellulose is obtained although in an impure condition containing pentosans.

Cellulose (crude fibre), free from pentosans, is obtained by König's process (*144*): 3 gms. of material are treated with 200 ccs. of glycerine of sp. gr. 1·23 mixed with 2% concentrated H_2SO_4, and either boiled under a reflux condenser at 133°–135° or treated with steam in an autoclave at 137° for one hour. After cooling, the mass is treated with water, boiled again, filtered hot, and washed with hot water, hot alcohol and a warm mixture of ether and alcohol until the filtrate runs out colourless. By treating the cellulose thus obtained with hydrogen peroxide and ammonia the lignin is oxidised and removed.[2]

Cellulose also dissolves in strong sulphuric acid (*e.g.* 72%), and yields large amounts of dextrose on boiling the diluted solution (*145*).

The process of Hoffmeister and König can be used for the quantitative estimation.

Lignin gives the reactions for woody tissues: red colour with phloroglucin and HCl, yellow colour with aniline chloride.

[1] A good solvent for cellulose is a solution of copper carbonate in ammonia; an aqueous solution of cupric sulphate is treated with the calculated quantity of sodium carbonate. The precipitate is filtered, washed well, and dissolved in concentrated ammonia.

[2] H. Ost and L. Wilkening, *Chemiker-Zeitg.* 94, 461, 1910; on the estimation of cellulose see also G. Fingerling, *Chemiker-Zeitg.* 46, 917, 1922.

Separation (and Estimation) of Cellulose in the presence of Mannans, etc., according to M. Lüdtke.[1]

Ten gms. of the powdered material, disintegrated by means of chlorine dioxide,[2] are treated with 1000 ccs. of 25% ammonia in a stoppered flask (capacity 1 litre), and then shaken with 15 gms. of cupric hydroxide introduced gradually, for fifteen hours. Carbon dioxide is then passed into the suspension until solution takes place. If the solubility point is passed by adding too much carbon dioxide it may be regained by the addition of copper hydroxide. Solution also takes place on the addition of ammonium carbonate or by decreasing the concentration of copper and ammonia. One hundred ccs. of 2*N* NaOH are then added, in small portions with shaking, to the centrifugalised, clear solution. The copper-alkali compounds of mannans are centrifugalised and washed once with alkali-ammonia solution (in the same proportion as before) upon the centrifuge. From the centrifugalised clear solution, as also from the wash-liquor, the cellulose is precipitated by 50% acetic acid, keeping the solution cool. The precipitate is centrifugalised, washed with 2% acetic acid until free from copper, rinsed with water, and finally dehydrated with methyl alcohol and ether.

XIV

PROTEINS

The preparation of pure proteins may be preceded in many cases by a mechanical separation from the accompanying matter. Thus the starch of wheat flour can be removed by washing and thereby separated from the protein matter (gluten). The crystalloids of a few seeds can be obtained by rinsing them out

[1] *Annal. d. Chem.*, 456, 201, 1927.

[2] About the disintegration with chlorine dioxide, see E. Schmidt and Graumann, *Ber. deutsch. chem. Ges.* 54, 1860, 1921 ; E. Schmidt and Malyoth, *ibid.* 57, 1835, 1924 ; M. Lüdtke, *loc. cit.* p. 208.

of the crushed seed kernels with ether, the ether rinsings combined, the suspended crystalloids poured out and allowed to settle, the ether decanted off, the crystalloids washed several times with ether, and the portion of ether adhering to the crystals removed by careful evaporation. In a similar way an oil or a mixture of an oil with petroleum ether can be used, the oil being again washed with ether or petroleum ether.

The investigation of vegetable proteins obtained in the above manner, or of those not yet extracted from the plant, depends upon the use of the same extracting or precipitating agents as are used in the preparation of the corresponding proteins from animal organs. As the proteins readily undergo changes, strong reagents which may alter their original state must be avoided.

The following are used for extraction : water, solutions of common salt of different strengths (5–10%), dilute solution of sodium carbonate (about 1%), dilute caustic alkalies (0·1–1%), and alcohol (40–80%).

The following summary shows the proteins which are dissolved by these liquids :

Soluble in water : vegetable albumins.

Insoluble in water, soluble in salt solutions : plant globulins.

Soluble in 70–80% alcohol : substances of the gliadin class.

Insoluble in neutral solvents, soluble in alkalies and precipitable from these by acids : those containing phosphorus : " vegetable casein ".

The usual methods of precipitation are the following : the proteins can be precipitated from their aqueous and salt solutions by boiling, treatment with alcohol, salting out with ammonium sulphate, magnesium sulphate and other salts or mixtures of these salts, often also by weak acids like acetic acid. The globulin-like proteins are precipitated from their salt solutions by dilution with water or by dialysis. The proteins are precipitated from weak alcoholic solutions by strong alcohol and from their solution in strong alcohol by ether.

The precipitated proteins are generally washed with alcohol

and ether, those soluble in alcohol are washed with ether, and then dried *in vacuo* over sulphuric acid.

The behaviour of proteins with extracting and precipitating agents may be used in addition to their chemical properties in assigning them to one of the groups so far distinguished. They have, however, the peculiarity that their physical properties may change without there being any change in the elementary composition. Such changes may take place even when the proteins remain under the prolonged action of water or alcohol, and more so when they are treated with (even weak) acids or alkalies.

These changes consist especially in the loss of their solubility in water or salt solutions. In addition to this the proteins readily undergo putrefaction. Decomposition of this kind can be prevented by working at a lower temperature or by adding a preservative like thymol or chloroform to the solutions.

If the plants were free from soluble salts the albumins and albumoses would be dissolved by extraction with water. But since plants always contain water-soluble salts, even if pure water is used the actual solvent is really a salt solution though it may be very dilute. Part of the globulin is, however, also soluble in such a solvent. It should also be remembered that plant extracts often have an acid reaction which may damage or alter the proteins for the reasons discussed above. A neutralisation of the extracts is therefore essential. This can be done by very dilute alkali or alkali carbonate, or, still better, by magnesium carbonate. In view of these considerations the investigation may be carried out in the following way :

The plant is extracted with 5–10% solution of common salt after the addition of a quantity of magnesium carbonate sufficient for neutralisation. A portion of the material thus completely extracted is treated with a solution of common salt and heated to 60°. It is then observed whether a precipitate of protein separates out on cooling. Many proteins will be precipitated on saturating the cold common salt solution with

common salt. Another separation of the globulins present in the salt solution can be effected by *Fractional Dialysis*, and a third method is that of *Fractional Coagulation* by warming.

As a rule fractional dialysis should be attempted first.[1] The globulin, which first separates out from the solution as it becomes gradually poorer in salts, is separated from the liquid, dialysed again, and so on until no more globulin separates out or the dialysing liquid becomes completely free from salts.

Each of the precipitated globulin fractions is dissolved separately in 10% NaCl solution and warmed carefully by putting the protein solution in a vessel containing water, the latter being placed in another vessel of water and heated from below. The proteins which separate out at one temperature are filtered off, the solution heated further, filtered again, and so on until the boiling point of water is reached. An attempt may also be made to determine whether different proteins can be isolated from the globulin solution by the fractional use of magnesium sulphate, ammonium sulphate or mixtures of these and similar salts. Each of the precipitates thus obtained is freed from salts by dialysis and then treated further with alcohol as described above.

It should next be ascertained by the general reactions for proteins (see p. 39) whether the liquid, freed from globulin, contains any more protein. If the liquid becomes red on application of the biuret reaction the presence of albumoses, which may be present in the liquid together with albumins, is indicated. The albumins are precipitated from the liquid by boiling and also by the addition of NaCl or $MgSO_4$ in an acid solution. The albumoses still present in the acid solution can be precipitated from it by means of zinc sulphate or ammonium sulphate.

After extraction with salt solution an extraction with alcohol may be attempted, since many of the vegetable proteins are

[1] The external fluid should be tested for albumoses, since these diffuse in very small quantities.

soluble in alcohol. 35–40% alcohol should be used first and then 75–80%. The proteins can be precipitated from the extract prepared with dilute alcohol either by concentration or by treatment with strong alcohol. The proteins soluble in strong alcohol can be finally precipitated by concentration or by ether. If alcohol-soluble proteins have been found the material should be extracted with alcohol first, since the proteins may partially lose their solubility in alcohol during the treatment with common salt.

As the next solvent a 1% solution of sodium carbonate may be used. It dissolves many proteins which have become insoluble, especially globulins, and allows them to be re-precipitated by neutralisation with weak acids or even by passing in carbon dioxide.

Lastly, extraction may be performed with weak (0·1–0·2%) KOH and the dissolved proteins be re-precipitated by neutralisation with acids.

A few vegetable proteins can be obtained in crystalline condition. Many globulins crystallise out when their solution in common salt is dialysed. Others crystallise out on cooling a warm solution. Schmiedeberg (*146*) treated the precipitated protein in a moist state with an excess of ignited magnesia and with water at 30°–35°. Crystals separate out from the filtered liquid when it is evaporated at a constant temperature of 30°–35°. The crystals are still better obtained, according to Drechsel (*147*), by keeping the filtrate containing the magnesia compound in a dialyser and putting this in absolute alcohol, whereby the water is slowly extracted from the solution.

Hofmeister obtained crystalline albumin by slowly evaporating its solution in a half-saturated solution of ammonium sulphate (*148*).

For thorough investigation of proteins it is necessary to hydrolyse them. Further particulars can be found in E. Fischer, *Ber. d. Dtsch. Chem. Ges.* 34, 433, 1901 ; 39, 530, 1906 ; E. Fischer, *Untersuchungen über Aminosäuren, Polypeptide und*

Proteine (Berlin 1906); Donald D. van Slyke, *Ber. d. Dtsch. Chem. Ges.* **44**, 164, 1911.

To separate the α-amino acids E. Cherbuliez distilled the acetyl derivatives of their ethyl esters at 1 mm. pressure (*148a*).

On the hydrolysis of proteins by enzymes see the work of E. Waldschmidt-Leitz and his co-workers, and also *Zeitschr. f. physiol. Chem.* **151**, 31; **156**, 68; *Ber. deutsch. chem. Gesellsch.* **59**, 3000, 1926; **60**, 359, 1927; *die Naturwissenschaften*, **14**, 129, 1926. Compare also E. Waldschmidt-Leitz and A. Schäffner, *Adsorptionsanalyse der Proteine und ihrer Abbauproducte*, *Ber. deutsch. chem. Gesellsch.* **60**, 1147, 1927.

Detection of Histidine in the Protein complex (H. Brunswik) (*149*)

If the diazo-reaction (due to the presence of tyrosine and histidine) is positive a sample of the substance is warmed with 20–50% HNO_3, some solid sodium carbonate is gradually added until the reaction is alkaline and the solution is divided into two parts. One part is tested with Millon's Reagent and the other with fresh *Diazo Reagent* prepared according to Pauly.[1] If the first reaction is negative and the second positive then histidine is present.

The method depends upon the fact that nitrated tyrosine, unlike unchanged tyrosine, does not give Millon's Reaction or the Diazo Reaction, whereas histidine is not sensitive to careful nitration.

The quantitative estimation of proteins is best done by carrying out the preparation quantitatively. The method mostly used in food chemistry, viz., the estimation of nitrogen by Kjeldahl's method and calculation of the quantity of proteins present by multiplying by a factor (usually 6·25), seldom gives

[1] 2 gms. sulphanilic acid, suspended in 3 ccs. of water and 2 ccs. of HCl, are treated slowly with a solution of 1 gm. of $NaNO_2$ in 2 ccs. of water. The precipitated diazobenzene sulphonic acid is filtered off and washed with a little water. It is used in a solution made alkaline with sodium carbonate.

an accurate result, even when the protein is separated from the other nitrogenous substances, according to Stutzer's process, by means of copper hydroxide.

XV

HYDROLYTIC PRODUCTS OF PROTEINS

A few nitrogenous substances, like amino-acids and their amides, occur in plants, especially in germinating seeds, as hydrolytic products of proteins. *Leucine*, *tyrosine*, *arginine*, *glutaminic acid* and *asparaginic acid* or *glutamin* and *asparagin* may be mentioned.

To prepare these substances (*150*) the crushed material is extracted with slightly warm water, the extract is purified by basic lead acetate (avoiding excess) and *asparagin*, *glutamin*, *arginine* and *tyrosine* are precipitated from the filtrate with a solution of mercuric nitrate. The precipitate is decomposed by H_2S, the filtrate neutralised with ammonia and evaporated at a temperature of 50°–60°. The liquid is kept neutral by the addition of a few drops of ammonium carbonate solution. Crystals separate from the evaporated solution on keeping *in vacuo* (in the case of arginin as nitrate). By taking up with a little cold water, the *tyrosine*, which is soluble with difficulty in water, can be separated from the other substances, the *arginine* being precipitated from the solution by phosphotungstic acid. *Glutamin* and *asparagin* are separated by taking up with a little cold water, in which the asparagin is less soluble than glutamin, evaporating the solution to crystallisation and separating the fine needles of glutamin from the granular crystals of asparagin by elutriation with the mother-liquor.

Asparagin and *glutamin* dissolve copper hydroxide on warming ; on cooling the hardly soluble copper compounds are precipitated.

Glutaminic acid can be separated from other substances, especially from asparaginic acid, by its zinc salt, which,

unlike that of asparaginic acid, is only soluble in water with difficulty.

Leucine is not precipitated by mercuric nitrate in sufficiently dilute solution and can be obtained by evaporating the filtrate previously treated with H_2S. By dissolving in alcohol leucine is separated from *tyrosine* when the latter has not been wholly precipitated by mercuric nitrate.

Leucine and tyrosine can also be separated by a mixture of glacial acetic acid and 95% alcohol by heating the substance with the mixture until it begins to boil; leucine goes into solution (*151*).

Besides these substances *lysine* and *histidine* come into consideration. Lysine and histidine are found together with *arginine* in the precipitate produced by phosphotungstic acid. The precipitate is decomposed by barium hydrate, freed from barium by carbon dioxide, and the filtrate is treated with a concentrated solution of corrosive sublimate (*152*) until the reaction is acid. The precipitate thus formed is decomposed by H_2S and the *histidine* is obtained as hydrochloride. The filtrate from the mercury precipitate is also treated with H_2S, and the liquid filtered from Hg is freed from H_2S by evaporation and treated with silver sulphate until the solution gives a yellow colour with NaOH. The liquid filtered from silver chloride is precipitated by baryta, and *arginine* is obtained from the precipitate by decomposition with H_2S. The filtrate from the arginine precipitate is acidified with sulphuric acid and freed from silver by H_2S. The liquid, filtered from $BaSO_4$ and silver sulphide, is exactly precipitated by baryta and the filtrate evaporated in order to crystallise the *lysine*, which can be distinguished by its almost insoluble picrate.

To isolate arginine A. Kossel and R. E. Grosz (*153*) treated the protein hydrolysate, obtained by boiling with sulphuric acid (33% by volume), from which most of the acid had been removed by lime or baryta, with an aqueous solution of flavianic acid = 1-naphthol-2, 4-dinitro-7-sulphonic acid (about four

parts of the acid for one part of the arginine expected). By frequent shaking, specially in the first hours after the addition of the precipitating agent, the formation of hard crusts of crystals is avoided, as these make it difficult to wash the precipitate.

The liquid is kept for three days in a cold place and the crystals are then filtered by suction.

For the purification of the precipitate and its conversion into the carbonate see the original work.

To separate histidine and arginine the dilute solution in sulphuric acid is treated with an excess of a hot, saturated solution of silver sulphate, and the solution brought to a p_H of 7·0 (6·8–7·2) by means of a cold saturated solution of baryta, whereby the silver histidine is precipitated. The precipitate is treated in hot water with a little HCl and the precipitation as silver salt is repeated. To precipitate the arginine the combined filtrate is concentrated *in vacuo* to about one and a half times the original volume, and the solution brought to a p_H of 10–11 by means of a warm saturated solution of baryta (*153a*).

On the separation of amino acids cf. also A. Kossel and J. Edlbacher, *Zeitsch. f. physiol. Chemie*, 110, 241, 1920.

Microchemical Detection of the most important α-mono-amino Acids, according to O. Werner (*154*)

The presence of an amino acid should first be tested for by the ninhydrin reaction and by precipitation with mercuric acetate and sodium carbonate. For the *Ninhydrin Reaction* the aqueous solution of a little of the substance is heated in a test-tube with a few drops of a solution of 0·1 gm. of ninhydrin in 40 ccs. of water. If present the liquid is coloured blue. To carry out the second reaction a little of the substance is dissolved on a slide in a larger drop of 10% aqueous solution of sodium carbonate and a very small drop of a 25% solution

of mercuric acetate is added. If an amino acid is present the granular precipitate is changed from yellow to white, and, if absent, it turns from yellow to reddish orange. The first test is more sensitive than the second.

The substance recognised as an amino acid is then submitted to vacuum sublimation (0·5 mg. will suffice) for which a special apparatus is used.

As further means of identification and separation of the amino acids the method of precipitation with phosphotungstic acid and the preparation of the copper salts may be used ; also the estimation of their capacities as solvents for the asparagin copper compound.

For details see *Mikrochemie*, **1**, 33, 1923.

XVI

PROTEINOGENETIC AMINES [1]

(including Choline and Acetyl-choline)

The proteinogenetic amines are partly volatile and partly non-volatile. The former can be distilled off with steam after the addition of alkalies. The distillate is either evaporated after the addition of HCl and the bases then precipitated from the concentrated solution as chloroaurate or chloroplatinate, or tartaric acid or oxalic acid is added and the base obtained as bitartrate or bioxalate, the latter, for example, in the case of isoamylamine. The non-volatile bases are generally obtained from the aqueous extracts by precipitation with metallic salts, seldom by shaking out of an alkaline solution. A more detailed description follows in which the methods are described for isolating *p*-oxyphenyl-ethylamine, imidazolyl-ethylamine, choline and acetyl-choline.

[1] By proteinogenetic amines are understood those amines which are derived by the splitting off of carbon dioxide from amino acids, the building stones of proteins. For more details on these substances see M. Guggenheim, *Die Biogenen Amine*, 2nd edition ; Berlin : Julius Springer, 1924.

p-Oxyphenyl-ethylamine (tyramine) was obtained by Barger (*155*) from ergot in the following way : the aqueous extract from 1·5 Kg. of ergot is concentrated *in vacuo* to 375 ccs. and shaken ten times with 150 ccs. of amyl alcohol after the addition of sodium carbonate : the amyl alcoholic extract is concentrated to 200 ccs. and shaken ten times with 30 ccs. of 1% aqueous NaOH. The alkaline solution is neutralised with HCl and evaporated. The residue is taken up with 250 ccs. of alcohol, and the solution treated with about 10 ccs. of a saturated alcoholic solution of mercuric chloride until there is no immediate precipitate. The filtrate is freed from alcohol first by evaporation and then by distillation in steam ; the filtered aqueous solution is treated with H_2S and, after filtering off the HgS, concentrated to 30 ccs. This solution is made alkaline with *N*/2 NaOH and shaken ten times with half its volume of ether.

The aqueous solution is neutralised with dilute HCl, made alkaline with a little sodium carbonate and shaken ten times with half its volume of ether, which now takes up the *p*-oxyphenyl-ethylamine and leaves it behind on evaporating the ether. For purification it may be converted into the dibenzoyl compound (M.P. 170°) by treating the solution, made alkaline by NaOH, with benzoyl chloride. The benzoyl compound can be recrystallised from alcohol and again hydrolysed by strong HCl.

p-Oxyphenyl-ethylamine $C_6H_4<^{OH\ (1)}_{CH_2\ .\ CH_2\ .\ NH_2\ (4)}$ forms hexagonal leaflets (M.P. 161°) which are coloured red with Millon's Reagent.

For the microchemical reactions of *p*-oxyphenyl-ethylamine and *β*-imidazolyl-ethylamine see L. van Itallie, and A. J. Steenhauer, *Microchemie*, III, 65, 1925.

To obtain imidazolyl-ethylamine (histamine) Barger and Dale (*156*) proceeded as follows : 500 ccs. of a dialysed extract of ergot were mixed with an equal volume of 20% solution of

tannin. The clear supernatent liquid was decanted off and freed from tannin by means of baryta water. The filtrate was freed from excess of Ba by H_2SO_4, and the last traces of tannin and sulphuric acid removed by freshly precipitated $Pb(OH)_2$. The filtrate was concentrated to 30 ccs., acidified with H_3PO_4 and mixed with 4 ccs. of 20% $AgNO_3$. After filtration more $AgNO_3$ solution (150 ccs.) was added until a drop of the solution gave a brown silver oxide with $Ba(OH)_2$. The solution was then treated with $Ba(OH)_2$ until a sample of the filtrate gave only a weak opalescence with ammoniacal silver nitrate. The precipitate was filtered, washed, suspended in dilute H_2SO_4 and decomposed with H_2S. From the filtrate the H_2S was removed, the solution neutralised and evaporated, and the residue extracted several times with hot alcohol. The residue from the alcoholic solution was dissolved in a little water and the solution treated with a hot saturated solution of picric acid. After a few days the picrate separated out and was recrystallised from water. It forms dark yellow rhombic plates which melt with decomposition at 234°–235°.

The free imidazolyl-ethylamine,

gives a red colour with diazobenzene-sulphonic acid in the presence of sodium carbonate.

Isolation of Choline and Acetyl-choline

(*a*) According to Ewins (*157*), repeated by Boruttau and Cappenberg (*158*): 1500 gms. of fluid extract, 1 : 1, were evaporated *in vacuo* to 400 ccs. and treated with aqueous mercuric chloride (1 : 16)[1] until no more precipitate was formed.

[1] The proteinogenetic amines are completely precipitated when the mercuric chloride solution is mixed with a saturated solution of sodium acetate (Engeland and Kutscher).

The filtrate was freed from excess of Hg by H_2S, the filtrate from HgS treated with a solution of sodium carbonate till it was only faintly acid, and then evaporated *in vacuo* to a thin extract. It was poured into 94% alcohol and the precipitate filtered off after standing twelve hours. The alcoholic filtrate with the wash-alcohol was distilled, the residue dried *in vacuo* and dissolved in 50 ccs. of methyl alcohol. (Any little residue remaining behind was washed with methyl alcohol and then removed by filtration). The methyl-alcoholic solution was precipitated with 300 gms. of absolute alcohol. An alcoholic solution of mercuric chloride (1 : 3) was added to the filtrate from this precipitate. After standing for several days the mercury precipitate was filtered, dried, finely powdered and lixiviated four times, each time with 150 ccs. of hot water, the residue being removed by filtration. The filtrate was cooled, again filtered, and the filtrate concentrated on the water bath to 40 ccs. The crystalline precipitate obtained thereby was filtered after cooling, dried, rubbed to a fine powder, suspended in water and decomposed with H_2S (this being repeated several times). The filtrate and warm water were freed from H_2S by a current of air and the strongly acid solution treated with freshly precipitated silver carbonate until the filtrate was free from Cl. The excess of Ag was removed from the filtrate by H_2S, the latter removed by air, the faintly alkaline solution neutralised with tartaric acid and a further amount of tartaric acid added equal to that necessary for neutralisation.

The weakly acid solution thus obtained was dried *in vacuo* at 60°–70° and the residue extracted completely with absolute alcohol. The alcoholic filtrate was concentrated to 15 ccs. and allowed to stand for two days ; the precipitated acid-tartrate of choline was then filtered. The alcoholic filtrate from choline tartrate was concentrated at 40°–50° and precipitated with alcoholic platinic chloride. The precipitate was lixiviated at 30°–40° with 70% alcohol, in which the acetyl-choline compound,

unlike that of choline, is easily soluble. On evaporating at 30°–40° the acetyl-choline-chloroplatinate is obtained (as slightly coloured, rectangularly crossed, needle-shaped crystals). The residue from the lixiviation with alcohol, which still contained considerable quantities of choline chloroplatinate, was dissolved in a little water and reprecipitated with three times the volume of alcohol.

(*b*) According to Boruttau and Cappenberg (*158*): 30 ccs. of fluid extract, 1 : 1, may be mixed with 70 ccs. of 95% alcohol, and the mixture precipitated completely with 1% alcoholic solution of platinic chloride. The precipitate is filtered after standing for one to two days, dried upon the filter and extracted first with a little cold water, which dissolves the choline chloroplatinate and leaves the acetyl choline compound undissolved. The latter is obtained by dissolving the residue in water and evaporating carefully. From the filtrate, which contains the choline chloroplatinate, the latter can be again precipitated by alcohol.

Choline, $(CH_3)_3 . N(OH) . CH_2 . CH_2 . OH$. Strongly alkaline syrup. Gives trimethylamine on heating with KOH. It gives crystalline precipitates, among others, with mercuric chloride, potassium bismuth iodide, picric acid and picrolonic acid.

If a dilute solution of choline perchlorate (about 0·1 gm. in 50 ccs. of water) is evaporated with 2 ccs. of a pure 65% solution of HNO_3 upon the water bath, and the residue is dissolved in a little water, the perchlorate of choline-nitric acid ester crystallises out after the addition of a few drops of dilute perchloric acid (*159*), in large leaflets with satin-like lustre.

Acetyl-choline, $(CH_3)_3N . (OH) . CH_2 . CH_2 . O . COCH_3$. This gives crystalline precipitates with gold chloride (granules or prisms agglomerating into warts or tree-like branched needles) and with platinic chloride (needle-shaped crystals in rough crosses or comb-like and needle-shaped forms).

XVII

ENZYMES

(*a*) **Detection**—The detection of a vegetable enzyme is considered to have been accomplished when the change in a certain reaction by the agency of a plant extract or preparation is distinctly greater than that to be expected from the stoichiometric relations between the quantity of enzyme and the reacting substance, and when this activity is destroyed by heat. The latter property often suffices for control.

The substance should be treated aseptically or with an antiseptic (toluene, thymol), and, if possible, adjusted to the optimum p_H.

1. **Enzymes, which hydrolyse carbohydrates or glucosides.** Since the hydrolysis of carbohydrates and glucosides is accompanied by changes in optical activity, and probably always also by the formation of reducing sugars, the polarimeter and estimations of sugar serve for detecting these enzymes (cf. p. 142). One may test for *saccharase* by its action on cane sugar, for *maltase* by its action on maltose, for *β-glucosidase* by action on β-methyl-glucoside or salicin, for *α-glucosidase* by action on α-methyl-glucoside, for *myrosinase* (myrosin) by action on sinigrin, etc. In the latter case mustard oil can also be detected in this way.

To test for *amylase* (*diastase*) 10 ccs. of the solution to be tested are kept with 1 c.c. of a 1% solution of soluble starch in a water bath at 45°. After one hour at the earliest 1 c.c. of iodine-potassium-iodide solution (1 : 2 : 300) is added and shaken. If there is no blue colour amylase is present.

2. **Enzymes, which hydrolyse esters (esterases)**—To ascertain the presence of an ester-splitting enzyme the preparation to be tested for the enzyme (or the crushed plant) is brought in contact with the ester, which should be either emulsified or in solution, and the acid set free is estimated.

To test for a *fat-splitting enzyme* (*lipase*) the material is shaken with 2·5 gms. of olive oil and 2 ccs. of acetate buffer ($p_H = 4{\cdot}7$) for three minutes; it is then kept in an incubator for some time. Thirty ccs. of 96% alcohol and 15 ccs. of ether are next added and the solution titrated with $N/10$ alcoholic KOH (Willstätter) (*160*). A control experiment is done without the enzyme. The acetate buffer consists of equal volumes of $N/2$ acetic acid and $N/2$ ammonium acetate.

If the enzyme to be detected is not a lipase, other esters, *e.g.* ethyl butyrate or amyl baldrianate, may be used. For *esterases, which hydrolyse phenol esters*, a test is made with guaiacol carbonate and peroxidase (from wheat bran). A red solution is obtained when positive, since the guaiacol set free is oxidised by the peroxidase to tetra-guaiacol-quinone.

3. **Proteoclastic enzymes** (proteases)—(1) The coagulated and disintegrated protein is mixed with the enzyme solution in a dialysing sack and dialysed in an incubator against 20 ccs. of water for 12–16 hours at 37° after covering both the solutions in the dialyser with a layer of toluol. 0·2 c.c. of a 1% aqueous solution of triketohydrindene hydrate (*ninhydrin*) is added to 10 ccs. of the dialysate and boiled for one minute. If positive a blue colour will be found (Abderhalden) (*161*).

(2) A petri dish is filled with ox- or horse-serum up to a height of about $\frac{1}{2}$ cm. and placed in a drying chamber at about 70° until the serum solidifies completely. A few drops of the enzyme solution, made neutral as nearly as possible, are then placed in the dish and left for twenty-four hours at 50°. When positive every drop produces a depression (Müller-Jachmann) (*162*).

(3) The acid nature of the product formed is demonstrated by titration with alkali in the presence of alcohol. For details on the subject see R. Willstätter and E. Waldschmidt-Leitz, *Ber. Deutsch. Chem. Gesellsch.* 54, 2988, 1921.

Rennet (*chymase*, *chymosin*) is indicated when the solution coagulates milk. Mustard oil has been used as an antiseptic.

4. **Amidases**—*Urease* is detected by allowing a solution of the preparation or the plant powder to react upon a 1% solution of urea to which a little phenolphthalein has been added. The test is positive when the liquid is coloured red. Optimum $p_H = 7{\cdot}3$ (approx.).

The solution may also be made alkaline after some time with sodium carbonate, and the ammonia formed carried by a strong current of air (aeration) into a wash-bottle containing acidified water and tested with Nessler's reagent, etc.

On the detection of *adenase* and *guanase* see G. Woker, *Die Katalyse* (Stuttgart, F. Enke, 1924), Part II. First Half, p. 443 ; on the detection of *nuclease* see the same, p. 453.

5. **Catalase**—An approximately 3% acid-free solution of hydrogen peroxide, obtained by diluting *perhydrol*,[1] is allowed to act upon the material. The evolution of oxygen (which is often violent) shows the presence of catalase. It often suffices to carry out the experiment in a test-tube or in a small flask. If the test-tube is provided with a glass tube passing through a cork the oxygen can be detected by the re-kindling of a faintly glowing piece of match-stick. The hydrogen peroxide can also be determined by titration (iodometric or oxidimetric). Optimum $p_H = 7$.

6. **Oxidases and Peroxidases**[2]—To demonstrate oxidases and peroxidases reagents are generally used which give strongly coloured oxidation products or products which will give colour reactions. Among others the following reagents are used :

Reagent.	Oxidation Product.
Guaiaconic acid (in 1% alcoholic solution) or a freshly prepared 5–10% alcoholic extract of guaiacum wood or an acetone extract of guaiacum resin.	Guaiacol-blue (can be shaken out with chloroform).

[1] E. Merck's perhydrol contains 30% H_2O_2 by weight.

[2] As oxidases one may understand here, putting aside all theoretical possibilities, those enzymes which possess oxidising properties without the addition of peroxides : peroxidases require peroxides.

Reagent.	Oxidation Product.
Aloïn (1% aqueous solution).	Aloïn-red.
Pyrogallol.	Purpurogallin.
Leuco-malachite green (0·1 gm. + 5 ccs. of glacial acetic acid + 495 ccs. of water).	Malachite-green.
Hydriodic acid (acidified solution of potassium iodide).	Iodine (shaken out with benzene or detected with starch paste).
Alkaline solution of *a*-naphthol + dimethyl-*p*-phenylene-diamine-hydrochloride.	Blue indophenol dyestuff.
Pyramidone (1% aqueous solution).	Bluish-violet substance.

Tyrosinase is detected by its action on tyrosine. When positive the solution of tyrosine becomes rose- and garnet-red, then brown and black.

7. **Asymmetrase**—The aqueous solution of the enzyme is shaken with 0·675 gm. of hydrocyanic acid and 5·3 gms. of benzaldehyde and after one to two hours it is shaken out with 25–30 ccs. of chloroform. After dehydrating the chloroform with anhydrous sodium sulphate it is tested polarimetrically. The solution is then poured out of the tube into a small flask, 25 gms. of fuming HCl are added and the mixture kept for half an hour with frequent shaking. The chloroform is distilled off, the residue rinsed with fuming HCl into a porcelain dish and evaporated as far as possible on the water bath. It is taken up with water and the aqueous solution (after clarifying with kieselgur) tested again with the polarimeter. The direction of rotation of the aqueous solution (due to mandelic acid) should be the reverse of that in chloroform solution (due to mandelic acid nitrile) (*163*).

(*b*) **Preparation**—The process to be used for the preparation of an enzyme depends upon the nature of the raw material and also upon the degree of purity which it is desired to attain.

The operations which come into consideration are (1) preliminary operations, (2) extraction, (3) separation, (4) purification.

The preliminary operations may consist of freeing the material from mechanical impurities by means of solvents like ether or alcohol which do not dissolve any enzyme. For further similar operations see Willstätter's process described below.

The extraction of the enzyme is done with water,[1] weak salt solutions, or 20% alcohol or glycerine, mostly at room temperature or lower, since enzymes in solution are sensitive to higher temperatures.

In order to exclude the action of bacteria and moulds, antiseptics such as chloroform or toluene or sodium fluoride should be added when extracting with water or salt solutions.

In order to liberate the enzymes from compounds which may be formed with cell-constituents it is often necessary to make the solvents slightly acid or alkaline. The use of stronger acids or alkalies should be avoided since most enzymes are very sensitive to these.

From the solution in water or glycerine or 20% alcohol the enzymes are precipitated by strong alcohol. It should, however, be remembered that a few enzymes are soluble in dilute alcohol and that the activity of some is impaired by longer contact with alcohol. Enzymes may also be precipitated from their solution in water or in dilute salt solution by saturation with ammonium sulphate.

The purification can be done in different ways, often by repeating the process of preparation. Many impurities may be removed by lead acetate or by mercuric chloride or by dialysis.

Electrodialysis and electro-osmosis may be used for the purification of enzymes. For details see R. Fricke and P. Kaja, *Ber. Deutsch. Chem. Gesellsch.* 57, 310, 1924.

Concentration may be effected in special cases by adsorbing the enzyme with a suitable substance and washing it out

[1] Special attention may be drawn to the fact that extraction with water alone does not always suffice to dissolve out the enzyme.

("elutriation") of the adsorbing material ("*adsorbate*"). This method has been recently developed by R. Willstätter and his pupils (cf. the preparation of invertase from yeast, *Annalen der Chemie*, 425, 1, 1921).

A somewhat purified **emulsin** is obtained by the process of K. Josephsohn :

200 gms. of placenta amygdalarum amararum are shaken in a shaking machine with 500 ccs. of 0·1 *N* ammonia for a few hours ; the substance is then separated in a large centrifuge (at approx. 2700 revolutions per minute) and the residue is shaken again with 500 ccs. of 0·01 *N* ammonia for two to three hours. The protein is precipitated by the addition of 110 ccs. of 0·5 *N* acetic acid, it is centrifugalised off together with any precipitates formed subsequently within a day, and the filtrate precipitated with three times its bulk of alcohol. For use the precipitate is dissolved in water, filtered, and the filtrate utilised.

A detailed method of purification is found in Wroblewski's process for **the preparation of diastase** (*164*). It depends upon the fact that diastase is soluble in 50% alcohol but insoluble in 65% alcohol.

Three kg. of malt are extracted for twenty-four hours with 6 kg. of 68% alcohol. The well-pressed residue is macerated for one day with 6 litres of 45% alcohol, the liquid filtered, the residue pressed out and extracted in the same manner. The last two macerations are combined and treated with 96% alcohol until the content of alcohol in the liquid amounts to 70%. The precipitate is allowed to settle and washed with 70% alcohol. It is then dissolved by rubbing in 6 litres of 45% alcohol, the enzyme is precipitated as before and then dissolved in as little water as possible. By saturation with magnesium sulphate it is again precipitated, washed with a concentrated solution of magnesium sulphate, dissolved again in the smallest possible amount of water and dialysed until the dialysate gives no precipitate with barium chloride. Finally, the enzyme is

precipitated with a mixture of alcohol and ether and dried *in vacuo*. The separation of the enzyme from any carbohydrate accompanying it is attained by fractionally precipitating the solution of the impure enzyme with ammonium sulphate.

As an example of Willstätter's process the method for the preparation of peroxidase from horse-radish by Willstätter and Stoll's method (*165*), and the preparation of *emulsin* by Willstätter and Csányi's method (*166*), may be described. The first depends upon dialysis in the plant material, adsorption also in the material through oxalic acid and fractional extraction with alkali.

The roots are kept in water for a day and cut into slices with a plane. Five kg. portions of the slices are submitted to dialysis in a stoneware vessel provided with inlet and outlet tubes for seven to nine days, allowing a flow of 100–150 litres of water per hour. The slices are then dried as far as possible by suction and digested with a solution of 30 gms. of oxalic acid in 15 litres of water with frequent shaking. The slices are filtered by suction, pressed out and then crushed in a rolling mill to a thin pulp. The pulp is mixed with 6–8 litres of water, filtered under suction and, without being allowed to dry, washed with 8–10 litres of 0·01% solution of oxalic acid. The mass is filtered by suction, pressed well, and stirred in a finely divided condition gradually with 1 litre of one-third to one-half saturated $Ba(OH)_2$. After half an hour's action (the reaction should remain faintly acid or neutral) it is pressed out, the press-cake disintegrated and stirred with $1\frac{1}{4}$ litres of $Ba(OH)_2$ saturated at 20°. After about half an hour's action it is strongly pressed, and the press-juice treated with CO_2 until it has a faintly acid reaction. The press-cake may be treated twice more in the same manner, each time with $\frac{1}{2}$ litre of half-saturated $Ba(OH)_2$. Each extract is neutralised with CO_2 and mixed with nine-tenths of its volume of 96% alcohol. The combined extracts are kept overnight in a cool room, decanted as far as possible and then filtered through a large smooth

filter. The residue is centrifugalised. From the filtrate the peroxidase is obtained by concentrating the liquid *in vacuo* [1] to 50–70 ccs. and then precipitating with five times its bulk of absolute alcohol. A purification is attained by treating the solution of 1 gm. of crude enzyme in 20 ccs. of water with H_2SO_4 until weakly acid in reaction and then precipitating with six times its volume of alcohol. From this preparation one can still separate an inactive glucoside by means of $HgCl_2$ and precipitate the peroxidase from the filtrate with alcohol. The precipitate is dissolved, without previous drying, in a little water, precipitated again after filtration, and the process repeated until the enzyme dissolves in water to a clear solution.

The preparation of emulsin can be done, according to R. Willstätter and W. Csányi, in the following way: Sweet almonds are warmed up in water at 60°–70°, skinned and dried superficially in the air. They are crushed in an almond mill and pressed in a hydraulic press. To free them completely from oils they are extracted in a flask with three times their bulk of ether, filtered by suction, powdered in a rolling mill, extracted again with double their bulk of ether, filtered by suction and washed. They are finally dried in a current of warm air and powdered finely in a mill.

100 gms. of this powder are shaken in a flask with 250 ccs. of $N/10$ ammonia, diluted with 100 ccs. of water and shaken for five hours in a shaking machine. The ammoniacal extract is best separated by a centrifuge, the residue shaken in a centrifuge glass with a mixture of 100 ccs. of water and 10 ccs. of $N/10$ ammonia, centrifugalised again and extracted a third time, should the alkaline reaction disappear, after the addition of a few ccs. of ammonia. The combined extracts are mixed with 300 ccs. of $N/10$ or 60 ccs. of $N/2$ acetic acid. The filtrate is then mixed with four times its bulk of alcohol, the precipitate separated by a centrifuge and washed with

[1] Bath temperature 50°, internal temperature of the flask, which is dipped only up to the level of its contents, 30°.

absolute alcohol and ether. For further purification of this preparation see *Zeitschr. f. physiol. Chem.* 117, 172, 1921.

For the preparation of a vegetable *perhydridase* see D. Michlin, *Biochem. Zeitschr.* 185, 216, 1927.

The separation of different enzymes from one another can also be done by means of an adsorbing substance. An example of this is found in the work of Waldschmidt-Leitz and A. Harteneck, *Zeitschr. f. physiol. Chemie*, 147, 286, 1925, and R. Willstätter, and his co-workers, *ibid.* 161, 191, 1926.

Maltase is obtained free from saccharase when it is isolated by a special kind of alumina (argillaceous earth), the adsorption compound containing only a very small amount of saccharase. The saccharase is first washed out at 0° with a primary phosphate and the maltase is then extracted with diammonium phosphate.

For details see R. Willstätter, and E. Bamann, *Zeitschr. f. physiol. Chemie*, 151, 273, 1925.

On the separation of enzymes by ultrafiltration see H. Bechhold and L. Keiner, *Biochem. Zeitschr.* 189, I, 1927.

On the isolation of enzymes from plants see also C. Oppenheimer and L. Pincussen, *Die Methodik der Fermente*, pp. 428 *et seq.*

XVIII

TOXALBUMINS

The vegetable toxalbumins are obtained partly like the proteins and partly like the enzymes. *Ricin* (*167*), for example, is prepared by extracting pressed Ricinus seeds with 10% NaCl in a percolator, saturating with $MgSO_4$ and Na_2SO_4 at room temperature and keeping the solution in a cold place. The white precipitate of toxalbumin is obtained together with the crystals of both the sulphates from which it is mechanically separable, and is filtered in the cold, placed in the dialyser without washing and dialysed.

XIX

INORGANIC CONSTITUENTS

The inorganic constituents of a plant are usually investigated by incinerating. This method, however, neither gives any exact information concerning the nature of the compounds originally present in the plant nor does it allow all the inorganic constituents of the plant to be detected. Ammonia and nitric acid are volatilised by heat, and HCl may be removed by the action of hot silicic acid upon the chlorides present. By the action of glowing charcoal upon the acid-salts of phosphoric acid phosphorus may be formed which will likewise volatilise. By the usual method of incineration a portion of the salts, *e.g.* the halides of alkalies, may also be lost by evaporation. The alkalies and alkaline earths, which are combined with organic matter, remain behind as carbonates. Sulphates and phosphates may be formed on incineration from the S and P present in organic combination. Sulphides and cyanides may likewise be found in the ash although they were not originally present in the plant.

If it is desired to obtain as far as possible an exact record of the inorganic constituents actually present and of their states of combination, one should first of all examine the extracts, obtained with water and dilute HCl, according to the methods of analytical chemistry,[1] and submit the carefully dried residue to incineration, keeping in mind, however, the changes which may occur on incineration mentioned above.

The incineration is usually done in platinum or porcelain dishes or in crucibles of the same material, which are either heated over the naked flame or in muffles. Alternatively, the *incineration apparatus of Tucker* (*168*) may be used. It offers the advantage of checking the volatilisation of inorganic constituents besides possessing a few other advantages.

[1] One should keep in mind that a few precipitates may be hindered by the presence of organic matter.

On a new oven for rapid incineration see A. Fornet, *Chemiker-Ztg.* 52, 319, 1928.

On incineration in a current of moist oxygen see S. Tausz and H. Rumm, *Chemiker-Ztg.* 49, 665, 1925.

To help the incineration the addition of various substances has been recommended, among which spongy-platinum, calcium plumbate, barium hydroxide, magnesium acetate and calcium acetate may be mentioned.

Instead of incineration the organic substances may be decomposed by HNO_3 and concentrated H_2SO_4 (*169*), by which method, however, some of the anions, *e.g.* $-SO_4''$, $-Cl'$, $-NO'_3$ may escape detection.

Of inorganic anions, one may find: $-Cl'$, $-Br'$, $-I'$, $-SO_4''$, $-NO_3'$, $-PO_4'''$, $-BO_3'''$, and also silicic acid. Of cations, $K^{\cdot}$, $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Fe^{\cdot\cdot}$, $Na^{\cdot}$, $Al^{\cdot\cdot\cdot}$, $Mn^{\cdot\cdot}$, $Zn^{\cdot\cdot}$, $Cu^{\cdot\cdot}$ may be mentioned.

For the detection and estimation of these substances the text books on analytical chemistry may be referred to. The detection of only a few substances, which often occur only in traces, may be treated here.

1. **Iodine** (Th. v. Fellenberg) (*170*).

The material (generally 1–3 kg.), in 100 gm. portions, is treated in a large flat iron basin with 7 gms. of calcium hydroxide ground to a fine paste and with 3 ccs. of a concentrated solution of potassium carbonate (850–900 gms. K_2CO_3 per litre [1]). It is diluted with water until the material is uniformly covered during the subsequent boiling. It is evaporated in a gas oven, dried and carefully incinerated. The best way is first to evaporate the liquid portion and then to add the solid. If woody or hard materials are to be incinerated it is advisable in certain cases to heat the substance with the alkali in an autoclave for half an hour at a pressure of two to three atmospheres.

[1] One should use the solution obtained by treating the K_2CO_3 with 60–80% alcohol in order to free it from iodine.

The material is freed from moisture by gentle heat and the temperature is gradually raised so that the material is not ignited. During this it is kept constantly stirred with a flat iron rod. Towards the end the rim of the basin is heated in order to decompose the crusts of organic matter adhering to it. When the incineration is complete as far as possible the ash is boiled five to six times with fairly large amounts of water and filtered each time by suction. The filtrate should be colourless or very light yellow in colour. The combined filtrates are evaporated almost to dryness in a glass flask.[1] The moist residue is extracted several times with alcohol. The solution is evaporated to dryness after the addition of two to three drops of the solution of K_2CO_3; the residue is ignited, and the ignited residue again extracted with alcohol. The solution is evaporated, the residue ignited carefully, dissolved in about 1 cc. of water and the solution placed in a small test-tube (inside diameter 5 mm., height 80 mm., capacity 2 ccs.; cut slantwise at the top), to which is added 0·02 cc. of chloroform and a drop of nitrite-sulphuric acid (about 0·05 gm. of KNO_2 to 10 ccs. of $3N$ H_2SO_4) by means of a micro-pipette. The chloroform is brought to the bottom by frequently knocking the tube, which is held vertically, and finally it is centrifugalised.

For quantitative estimation by this process see *Ergebnisse der Physiologie*, XXV. p. 207.

To detect the presence of iodine in fats and oils 100 gms. of the substance are saponified by heating with 10 gms. of KOH, dissolved in very little water and 50 ccs. of 95% alcohol for half an hour under a reflux condenser. The alcohol is distilled off and the soap taken in an iron basin. For each 100 gms. of

[1] If too much material has been incinerated, or if the solution begins to froth during evaporation, it is neutralised (phenolphthalein) with 12½% HCl until it becomes almost colourless. The solution is evaporated further and the neutralisation is repeated from time to time, since the solution again tends to become red. Towards the end the solution is evaporated, slightly alkaline, to a paste and treated further as before.

If the material is rich in silicic acid the neutralisation is essential, as otherwise sodium-silicate goes over to the alcoholic solution and vitiates the experiment.

fat 5 gms. of dissolved lime are added, and heated until dry distillation of the soap takes place, ignition being as far as possible prevented. Towards the end, the material is ignited and the edges specially heated and then washed out. Any remaining carbon is burnt out. The remainder of the process is the same as above.

2. **Bromine.** The material is incinerated after the addition of lime, dissolved in water, and neutralised ; ferric ammonium sulphate is added and boiled in order to remove the iodine. The bromine is then set free by the addition of potassium dichromate and sulphuric acid, and is distilled over into 5 ccs. of a 0·025% solution of flourescein. As soon as the first trace of bromine comes over the flourescein solution shows the distinct reddish tint of eosin, and the colour and spectrum of flourescein disappear more and more ; the spectrum of eosin, with a sharper absorption band between E and b, becomes visible (*171*).

3. **Boron.** The ash is boiled with a solution of sodium carbonate, concentrated, and the filtrate acidified with HCl. A drop of the liquid is allowed to evaporate at the end of a thread of turmeric.[1] If a brown colour is observed under the microscope (with condenser illumination) it is brought into contact with a drop of a 13% solution of sodium carbonate. In the presence of boric acid a transient blue colour is obtained (*172*).

4. **Copper.** The material is first incinerated, the ash is extracted with HCl and $KClO_3$, the excess of chlorine and HCl is removed by evaporation to dryness and the residue taken up with water. A few drops of this solution are added to a tincture of guaiacum containing a little hydrocyanic acid (or

[1] To prepare the thread, 5 gms. of turmeric powder are boiled with 10 gms. of alcohol, filtered, and the solution evaporated. The residue is dissolved, with the addition of some sodium carbonate, in a few ccs. of 50% alcohol and boiled, and threads of unbleached linen are drawn through it. After taking them out they are pressed between filter papers, placed in very dilute sulphuric acid and washed with water (Emich).

better an alcoholic solution of guaiaconic acid) or a solution of aloïn. If there is no blue colour in the first case and no red colour in the latter case then the presence of copper is excluded.

Other very sensitive tests for copper are those with 1, 2-diamino-anthraquinone-3-sulphonic acid (bluish-violet colour in a solution made alkaline with NaOH) and with benzoin oxime (green precipitate). A drop of the solution to be tested is placed upon the slide and a drop of a 5% alcoholic solution of benzoin oxime allowed to flow into it. In the presence of copper, green streaks or, when a small drop of glycerine is added, micro-crystals are obtained (*173*).

5. **Manganese.** When manganese is present the ash is coloured green. Even if it is not coloured the ash is dissolved in HNO_3 and the solution warmed after the addition of ammonium persulphate [1] and a little silver nitrate. If manganese is present the permanganic acid formed colours the liquid violet-red. Permanganate is also formed if the acid solution is heated with NaOH and a little copper sulphate and then bromine water are added.

6. **Aluminium.** (i) The acid solution to be tested is treated with one-fifth of its volume of a 0·1% solution of alizarin-reds [2] and then with ammonia until there is a purple colour. It is boiled for a short time, cooled, and acidified with dilute acetic acid. In the presence of aluminium there is a red precipitate or a red colour (Atack) (*174*). One may also add only one drop of the alizarin solution and allow the sample to stand for two to three days (if necessary) after the addition of acetic acid (Willstätter, Kraut and Wenzel) (*175*).

[1] Silver peroxide nitrate, $2Ag_2O_2 . AgNO_3$ works still better (A. Travers, *Compt. rend.* 182, 1088, 1926, according to *Zeitschr. Analyt. Chem.*, 71, 130, 1927).

[2] Instead of alizarins one may use a solution of 0·5% alizarin in NaOH. The solution to be tested is treated with 1 cc. of the reagent, then acidified with 5% acetic acid and saturated with ammonia. In presence of aluminium a purple red colour and precipitate are obtained.

(ii) The solution in mineral acid is treated with sodium acetate, then with acetic acid, and finally with a 0·1% solution of morin in 20% alcohol. A greenish fluorescence (Goppelsröder) (*176*) is obtained, the sensitiveness being especially increased by Tswett's Luminoscope (Schantl) (*177*).

For both these reactions one may use the solution of the ash in HCl directly. Larger quantities of iron, which interfere with the reaction, can be made harmless in Atack's reaction by the addition of citric acid, and in Goppelsröder's reaction by filtering through a filter candle. In both cases one can also naturally use an HCl solution of aluminium hydroxide by the usual analytical methods.

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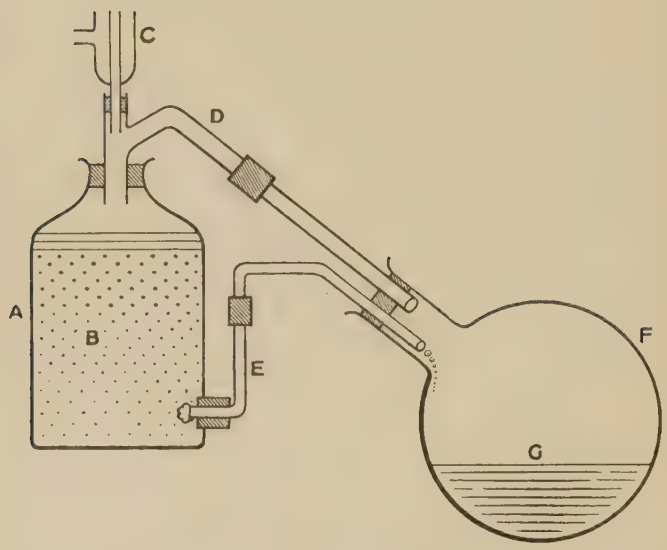
APPENDIX



WESTER'S EXTRACTION APPARATUS

(See *Anleitung zur Darstellung phytochemischer Übungspräparate*, by Dr. D. H. Wester, 1913, p. 12.)

A = Extractor (an aspirator bottle whose size may be varied according to material).

B = Material (coarsely powdered).

C = Condenser (cooled with ice-water if necessary).

D = Side tube (wider than syphon tube).

E = Syphon tube (the lower end being loosely closed with linen).

F = Flask (placed over a safety water bath or steam bath).

G = Solvent.

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